Department of Immunology, the Wenner-Gren Institute
Stockholm University, Stockholm, Sweden

Doctoral thesis
Stockholm, 2008

Host genetic factors and antibody responses with potential involvement in the susceptibility to malaria

Elisabeth Israelsson

If you think you are too small to matter,
you have never been in bed with a mosquito
Betty Reese

Distributed by Stockholm University Library. Printed in Sweden by Universitetsservice AB, Stockholm 2008

ISBN 978-91-7155-763-6 pp 1-84

Summary

The relatively lower susceptibility to malaria seen in the Fulani ethnic group in Africa, as compared to other sympatric ethnic groups, has been related to genetic regulation of the immune responses. This thesis aimed to describe important pathways related to the regulation of antibodies in the complex immune responses during a malaria infection.

Our results suggest that the higher anti-malarial immune responses seen in the Fulani, are not due to a general hyper-responsiveness in this group, but neither a malaria specific response. Rather, the higher responses in the Fulani are pathogen related, certain antigens/pathogens being more immunogenic in the Fulani ethnic group. Moreover, it appears as if the IgG subclass pattern to a malaria antigen differs between the Fulani and the non-Fulani groups, and certain polymorphisms in some of the Fcγ receptor genes showed a possible influence on the IgG subclass levels, as did some cytokine polymorphisms. These results suggest that the levels of IgG subclasses may be genetically regulated and that differences in these genes could influence the susceptibility to malaria.

Fcγ receptors are important structures in the humoral immune responses, and FcγRIIa exhibits a polymorphism at position 131 R/H. The 131H allele was associated with the Fulani group, and the 131R allele with the non-Fulani group. The FcγR 131 R/H also associated with IgG subclass levels and parasitemia, suggesting that this polymorphism may be a contributing factor to the differential susceptibility to malaria. No consistent interethnic differences were obtained for the other Fcγ receptor polymorphisms. However, associations with haemoglobin levels and *P. falciparum*-reactive IgG subclass antibodies were observed. This suggests an influence of these polymorphisms on malaria severity rather than the susceptibility.

C-reactive protein levels rise immediately in response to inflammatory stimuli. However, the net-effect of CRP is anti-inflammatory, due to the up-regulation of IL-10, which may affect parasite clearance and pathology. We investigated three CRP gene promoter polymorphisms, related to the circulating levels of CRP, and the -717T allele and the -286A allele were found to be more common in the non-Fulani groups. These alleles have both been linked to higher circulating CRP levels, however only the -286 polymorphism was indicated to influence parasite levels, and having consistent result in two independent cohorts from two countries with a marked difference in malaria endemicity in our study. These results suggest that the -286 CRP polymorphism may be involved in the lower susceptibility to malaria seen in the Fulani ethnic group.

Several cytokines are important in maintaining the optimal parasite-neutralizing milieu in the host, and we investigated polymorphisms in some of these cytokine genes, in order to establish a possible influence of these on malaria susceptibility. The frequency of the high-producing IL-1β and the low producing IL-10 haplotypes were more frequent in the Fulani ethnic group as compared to non-Fulani individuals, suggesting a higher inflammatory response in the Fulani. Moreover, several of these haplotypes showed associations with haemoglobin levels, IgG subclass antibody levels and parasitemia, suggesting that IL-1β, IL-6, IL-10 and TNF could affect the susceptibility to malaria and the severity of the malaria infection.

Taken together, these data suggest that genetic factors have the ability to affect the antibody responses, and that several pathways can be affected. Moreover, the Fulani have a genetic predisposition for a higher inflammatory response during a malaria infection, which could lower their susceptibility to the disease. However, the control measures for this inflammation still have to be established and evaluated.

"The difference"

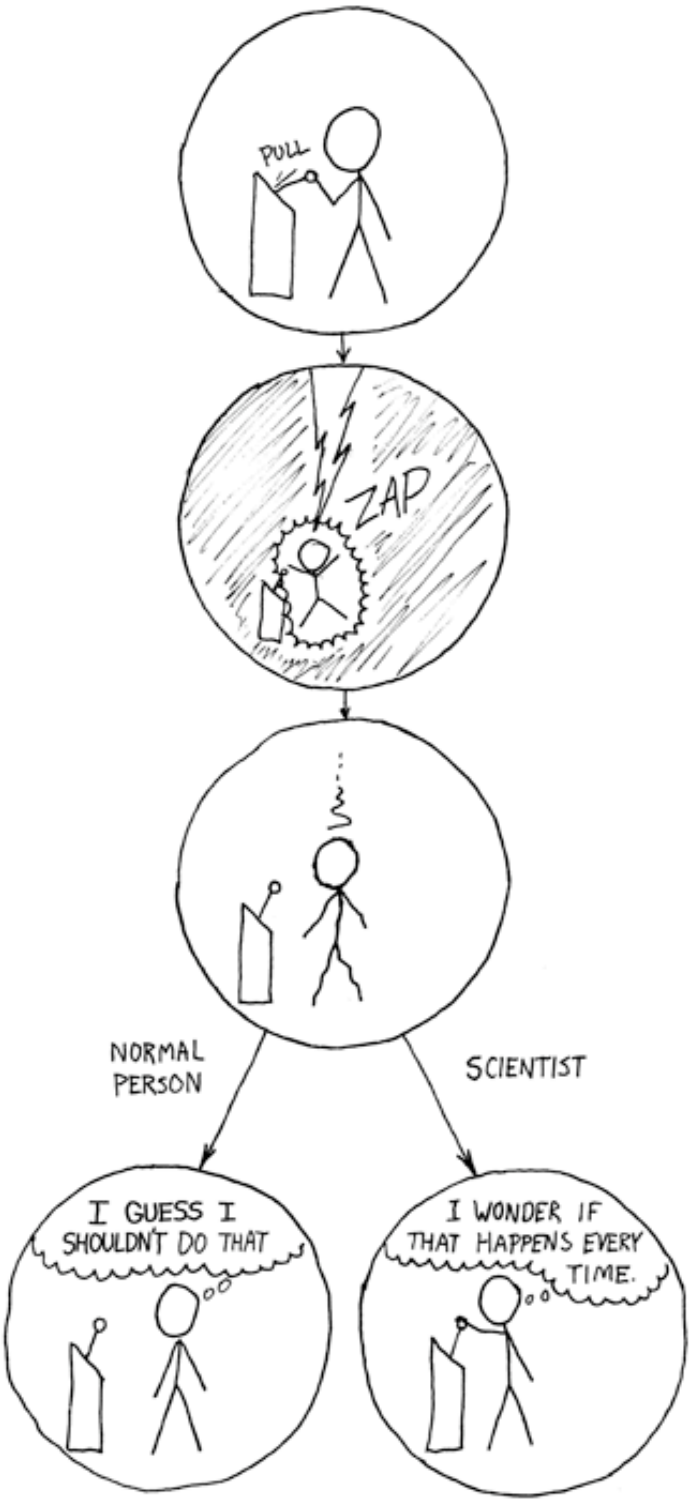


Table of contents

SUMMARY ..... **III**
TABLE OF CONTENTS ..... **V**
LIST OF INCLUDED PAPERS ..... **VII**
ABBREVIATIONS ..... **VIII**
INTRODUCTION **11**
INNATE AND ADAPTIVE IMMUNE RESPONSES 11
Innate immune functions *12*
Pattern recognition receptors 12
Cell of the innate immunity 12
The acute phase response *14*
C-reactive protein 16
Adaptive immune cells *19*
Antigen presentation 21
Immunoglobulins 22
Fc receptors 24
Cytokines *25*
Interleukin 1β 26
Interleukin 6 26
Interleukin 10 27
Tumour necrosis factor 27
Interferon-γ 27
MALARIA 28
History of malaria *28*
Prevalence, severity and impact on world health *29*
Malaria parasites and life cycle *30*
Malaria disease *32*
MALARIA IMMUNOLOGY 33
Naturally acquired immunity to malaria *33*
A proper immune response against malaria 33
Development and duration 34
The importance of antibodies in protection against malaria *35*
Important antigens 37
Host genetics and protection against malaria *38*
Red blood cell variations 39
Ethnic differences in genetic background 39
Polymorphisms in Fcγ receptor genes 40
C-reactive protein gene polymorphisms 41
Cytokine gene polymorphisms 42
Effects of co-infection with helminths, viruses and bacteria *43*
THE PRESENT STUDY **45**
AIM OF THE THESIS 45
MALI 47
Country profile and health status *47*
The malaria situation in the study area *49*
SUDAN 50
Country profile and health status *50*
The malaria situation in Daraweesh *52*
COMMENTS ON METHODOLOGY 53
Pyrosequencing™ *53*
Taqman® MGB probes *53*
Statistics *54*

Hardy-Weinberg equilibrium 54
Non-parametric tests 54
Parametric tests 55
Correction for multiple tests 55
Regressions 55
Association analysis using UNPHASED 55
Ethical permissions *56*
RESULTS AND COMPLEMENTARY DISCUSSIONS 57
Antibody levels and patterns of malaria reactive antibodies in relation to malaria susceptibility *57*
Genetic variations and ethnicity *59*
FcγR polymorphisms: are they relevant for malaria susceptibility? 59
CRP – a potent player in malaria susceptibility? 61
Can cytokine gene variations be a factor in malaria susceptibility? 61
Haplotypes 63
Biological associations of the genetic variations *65*
CONCLUDING REMARKS 67
FUTURE PROSPECTS 70
ACKNOWLEDGEMENTS 71
REFERENCES 74

List of included papers

This doctoral thesis is based on the following original papers, which will be referred to by their roman numerals:

I. **Distinct interethnic differences in IgG class/subclass and IgM antibody responses to malaria antigens but not in IgG responses to non-malarial antigens in sympatric tribes living in West Africa.** Ahmed Bolad,* Salah Eldin Farouk,* Elisabeth Israelsson, Amagana Dolo, Ogobara K. Doumbo, Issa Nebié, Boubacar Maiga, Bourema Kouriba, Gaia Luoni, Bienveu Sodiomon Sirima, David Modiano, Klavs Berzins, Marita Troye-Blomberg.
Scand J Immunology 2005 Apr; 61(4): 380-6
*These authors contributed equally to this paper.

II. **Differences in Fcγ receptor IIa genotypes and IgG subclass pattern of anti-malarial antibodies between sympatric ethnic groups in Mali.** Elisabeth Israelsson, Manijeh Vafa, Bakary Maiga, Anna Lysén, Nnaemeka C. Iriemenam, Amagana Dolo, Ogobara K. Doumbo, Marita Troye-Blomberg, Klavs Berzins.
Malaria Journal 2008, 7:175

III. **Fcγ-receptor genotypes in two ethnic groups showing different susceptibility against malaria in Mali, West Africa.** Elisabeth Israelsson, Susannah Kearsley, Manijeh Vafa, Bakary Maiga, Amagana Dolo, Ogobara K. Doumbo, Marita Troye-Blomberg, Klavs Berzins
2008 Manuscript

IV. **Marked differences in CRP genotype frequencies between the Fulani and sympatric ethnic groups in Africa.** Elisabeth Israelsson, Mattias Ekström, Amre Nasr, Amagana Dolo, Susannah Kearsley, Gishanthi Arambepola, Manijeh Vafa, Bakary Maiga, Ogobara K. Doumbo, Gehad ElGhazali, Hayder A. Giha, Marita Troye-Blomberg, Klavs Berzins, Per Tornvall
2008 Submitted

V. **Cytokine polymorphisms; influences on C-reactive protein levels, and a possible influence on malaria susceptibility.** Elisabeth Israelsson, Susannah Kearsley, Bakary Maiga, Amre Nasr, Amagana Dolo, Ogobara K. Doumbo, Gehad ElGhazali, Hayder A. Giha, Marita Troye-Blomberg, Per Tornvall, Klavs Berzins
2008 Manuscript

Abbreviations

ADCC	Antibody dependent cell cytotoxicity
ADCI	Antibody dependent cell mediated inhibition
APC	Antigen presenting cell
ATP	Adenosine triphosphate
CD	Cluster of differentiation
CRP	Complement-reactive protein
CSA	chondroitin sulphate A
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EIR	Entomological inoculation rate
Fab	Fragment antigen binding
Fc	Fragment crystallisable
FcR	Fc receptors
HWE	Hardy-Weinberg equilibrium
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iRBC	Infected red blood cells
ITAM	Immunoreceptor Tyrosin-based Activation Motifs
ITIM	Immunoreceptor Tyrosin-based Inhibiting Motifs
LPS	lipopolysaccharide
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MSP	Merozoite surface protein
NA	Neutrophil Antigen
NK cell	Natural Killer cell
NKT	NK T cell
PAM	Pregnancy associated malaria
PCR	**P**olymerase chain reaction
PRR	Pattern recognition receptors
RBC	Red blood cells
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TD	Thymus dependent
TGF	Transforming growth factor
Th	T helper
Th3	T helper 3 regulatory T cell
TI	Thymus independent
TLR	Toll like receptor
TNF	Tumour necrosis factor
Tr1	CD4+ Treg type 1
Treg	Regulatory T cells
VSA	Variant surface antigen

Amino Acid	**3-Letter**	**1-Letter**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Ju mer man tänker, desto mer inser man att det inte finns något enkelt svar.
Nalle Puh – AA Milne

Introduction

Innate and adaptive immune responses

The immune system is a highly variable and diverse component in all higher-animals. It has evolved throughout the years, and its complex network of cells and molecules can distinguish between invading pathogens and the body's own cells. Traditionally, the immune responses raised to an invading pathogen are divided into innate immune responses and adaptive immune responses. However, nowadays it is clear that these two parts are collaborating intensely and this division is now used to simplify rather than classify.

The innate immune system is a less specific first line of defence, and it involves anatomical barriers (skin and mucosal surfaces), physiological barriers (temperature, pH and chemical mediators), endocytic and phagocytic cells (monocytes, macrophages and neutrophils), and inflammatory responses. It reacts immediately in response to an invading microbe and the discovery of toll-like receptors (TLR) and their ability to recognise microbial molecules made it clear that innate immune cells can act in response to specific targets. Furthermore, these cells may also guide the adaptive immune responses, since two of the professional antigen presenting cell (APC) types, dendritic cells and macrophages, are innate cells with stimulatory effects on the adaptive immune response.

Clonally distributed B and T cells mediate the adaptive immunity and it exhibits specificity, diversity and memory. Small differences between pathogens can be distinguished, and a unique response will be raised against all particular antigens. Once the antigen has been recognized and responded to, an immunological memory will be developed, which by the next encounter with the same antigen will yield a faster immune response. The disadvantage with the adaptive immune system is that the primary response is delayed, due to the clonal expansion, however the following encounters with the same antigen will give a fast and specific immune response.

Innate immune functions

The innate immune cells are cells that do not rearrange their germline DNA to gain specificity. However, they do express a number of receptors that will increase the specificity of the cells, e.g. TLR and Fc receptors (FcR).

Pattern recognition receptors

The stimulation of the innate immune system is mainly mediated through pattern recognition receptors (PRR), which recognize conserved molecular structures found in large groups of pathogens. The TLR are the best characterized PRR family and thirteen TLR in mammals are known [1]. TLR1-9 are found both in humans and in mice, TLR10 is only found in humans, and TLR11-13 are only found in mice [2]. TLR can be expressed both on the cell surface (TLR1, 2, 4, 5 and 6) and in intracellular compartments (TLR3, 7, 8, 9), and they recognize different ligands, roughly covering all pathogens, the most studied being TLR4, recognizing bacterial lipopolysaccharide (LPS). Many cells depend, more or less, on TLR for their activation or functions. These receptors are important for dendritic cells (DC) maturation and function [3], they can amplify the antibody production and the memory maintenance in B cells [4] and it has been suggested that TLR could contribute to the maintenance and activation of T cell memory [5].

Cell of the innate immunity

Natural killer cells (NK cells) have the ability to react with spontaneous cytotoxicity, without sensitisation, against a broad range of targets, and they are also one of the key producers of cytokines that will mediate the immune responses of the other immune cells [6]. NK cells express a variety of receptors to induce their activities, including the natural cytotoxicity receptor family, the C-type lectin-domain containing receptors, the cluster of differentiation (CD) 2 superfamily receptors and the IgG receptor Fcγ receptor III (CD16) [7]. In addition to these activating receptors, the NK cells also express several families of inhibitory receptors, with the highly polymorphic killer cell immunoglobulin-like receptors (KIR) being the most well known.

NK cells can act as regulators on other cells, such as DC, T cells, B cells and endothelial cells. NK cells can kill the cells, but the cytotoxic killing of target cells can also induce antigen specific adaptive responses [8]. Few years ago, it was proposed that NK cells could have

long-lived and antigen-specific memory qualities in mice [9], and also that there is a regulatory NK cell population [10], whether or not this is true for humans needs further studies. NK cells secrete high amounts of interferon-γ (IFN-γ) that can promote the priming of T helper (Th) cells, but can also kill the cells if not major histocompatibility complex (MHC) class I is expressed in a sufficient amount by the target cell. The various aspects of NK cell functions make it a potent player in many different biological events, such as in the elimination or control of viruses, bacteria, parasites and tumours, in asthma and autoimmune diseases, as well as in the remodelling of the uterine spiral arteries and in organ transplantations [8]

Mononuclear phagocytes are represented by monocytes and macrophages. Monocytes are heterogenic myeloid APC precursors, originating from the bone marrow, varying both in size and granularity. When released in the periphery, they circulate for several days before they are recruited to various tissues, where differentiation into macrophages or dendritic cells will occur. Monocytes express a high frequency of TLR, but also FcγR I-III to different extents. This makes the monocytes very prone to be activated both by microbial stimuli and by pathogen reactive antibodies.

Most macrophages are residing in the tissues, but some are still motile, moving in an amoeba-like manner throughout the tissues. Depending on the tissue location, the names may vary: alveolar macrophages in the lungs, Kupffer cells in the liver and mesangial cells in the kidney, to mention a few. Macrophages are divided into classically activated (M1), influenced by endogenous inflammatory stimuli by IFN-γ or by exogenous inflammatory stimuli by LPS, and alternatively activated (M2), activated by Th2 cytokines. However, this division is not static; M1 and M2 can be re-polarized by Th2 or Th1 cytokines respectively [11]. Activated macrophages have a more efficient phagocytic activity and an increased killing capacity, a higher expression of MHC class II and thereby an increased ability to activate T cells, and an increased secretion of inflammatory mediators.

Dendritic cells are characterized as the most potent APC, due to their abundant expression of MHC class II and co-stimulatory molecules. There are two conventionally described DC subsets in humans, the myeloid DC, originating from monocytes arising from the myeloid pathway, and the plasmacytoid DC, originating from the lymphoid pathway. These two subsets differ in their expression of TLR, but both are equally efficient in stimulating naïve T cells [12]. The antigen presentation is a very important aspect of the role of DC, making the

DC bridge the innate and adaptive immune responses. Recently a new subset of cytotoxic DC was described. These DC are called killer-DC and they are found both in rodents and in humans, however it is still unclear if these killer DC have an active function in humans [13].

Granulocytes are cells with a granulated cytoplasm. The most prevalent among them are neutrophils, a phagocytic cell with a multi-lobed nucleus, and this cell type is usually the first cell to arrive at the site of infection. Activated neutrophils will limit the infection by phagocytosis, by release of antimicrobial peptides and pro-inflammatory cytokines. They will also recruit and activate other immune cells and raise an adaptive immune response [14]. Therefore neutrophils are considered very important first line defenders.

Eosinophils are also phagocytes and a hallmark sign of helminth infections. They have been suggested to play an important role in combating parasitic infections, by secreting the contents in their granules that may damage the parasitic membranes. The major basic protein (MBP) accounts for more than 50% of the granule mass, and this has shown toxicity against parasites *in vitro* [15]. In certain inflammatory situations, eosinophils can function as a fully functional APC able to activate naïve CD4+ T cells [16].

Basophils are nonphagocytic cells that play a role in allergic and parasitic responses. Basophils rapidly produce large amounts of interleukin (IL) -4 in response to both allergen and parasites, and they are known to play a role in the rejection of ticks [15].

Mast cells are tissue based, with a heavily granulated cytoplasm, and they contain histamine and other active substances, which make them very important in allergic responses. Mast cells are producing many cytokines, the major one being tumour necrosis factor (TNF), but also IL-4 and IL-6 [15]. Mast cells may also be important for the suppressive function mediated by regulatory T cells [17].

The acute phase response

The acute phase response is a group of physiologic changes that occur shortly after the onset of an infection or other inflammatory processes, and it includes concentration changes of different plasma proteins, so called acute-phase proteins, and behavioural, physiologic, biochemical and nutritional changes. The definition of acute-phase proteins is, a protein whose concentration increases or decreases, depending on if it is a positive or negative acute-

phase protein, by at least 25 percent during an inflammation [18]. Positive acute-phase proteins are represented by complement proteins, coagulation and fibrinolytic proteins, antiproteases, transport proteins, inflammatory response participants and other proteins such as complement-reactive protein (CRP), serum amyloid A, ferritin and fibronectin [18], while negative acute-phase proteins are albumin, transferrin, transthyretin, α_2- HS glycoprotein, α-fetoprotein, thyroxine-binding globulin, insulin-like growth factor I, retinol-binding protein and factor XII [18]. The magnitude of the increase/decrease in concentrations varies with the different proteins, with the highest increase being seen in C-reactive protein and serum amyloid A (fig. 1). These proteins can increase 1000-fold their concentrations during an inflammatory response [18].

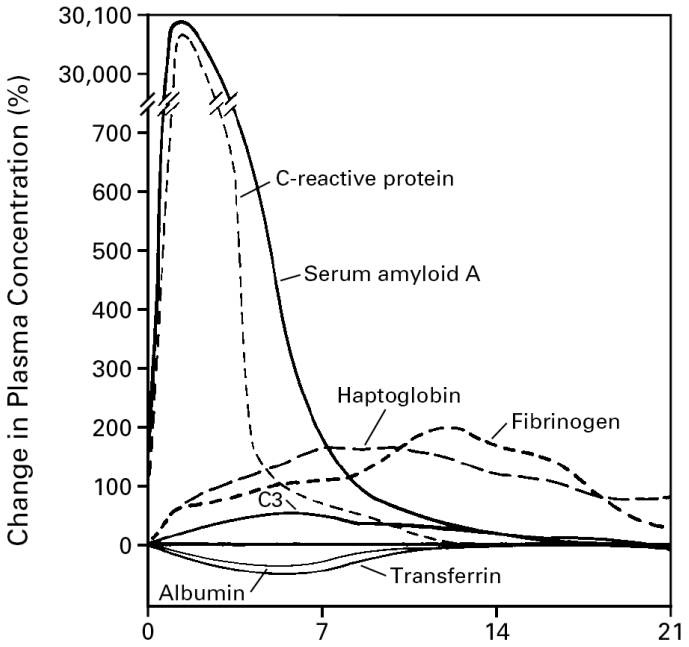


Figure 1. The characteristic pattern of the increase and decrease in some acute-phase proteins after a moderate inflammatory stimulus. Adapted from Gabay and Kushner 1999 [18]

The regulation of acute-phase responses is dependent mainly on cytokines produced during and/or participating in the inflammatory response. IL-6 is the most potent inducer, whereas the other cytokines, IL-1β, TNF, IFN-γ, and transforming growth factor (TGF)-β, are rather influencing subgroups of acute-phase proteins [18]. The initiation and progress of the acute-phase response involves a coordinated set of events, cytokine release, endothelial-cell activation, leukocyte chemotaxis and alterations of the temperature (fig. 2). Platelets and mononuclear phagocytes at the site of damage release early cytokines (IL-1, IL-6 and TNF), which are involved in the fever progress and the activation of other cells [19]. These activities will elicit a production of chemotactic cytokines and an accumulation of inflammatory cells,

which will activate the hepatic response, that results in an stimulation of acute-phase proteins and responses [19].

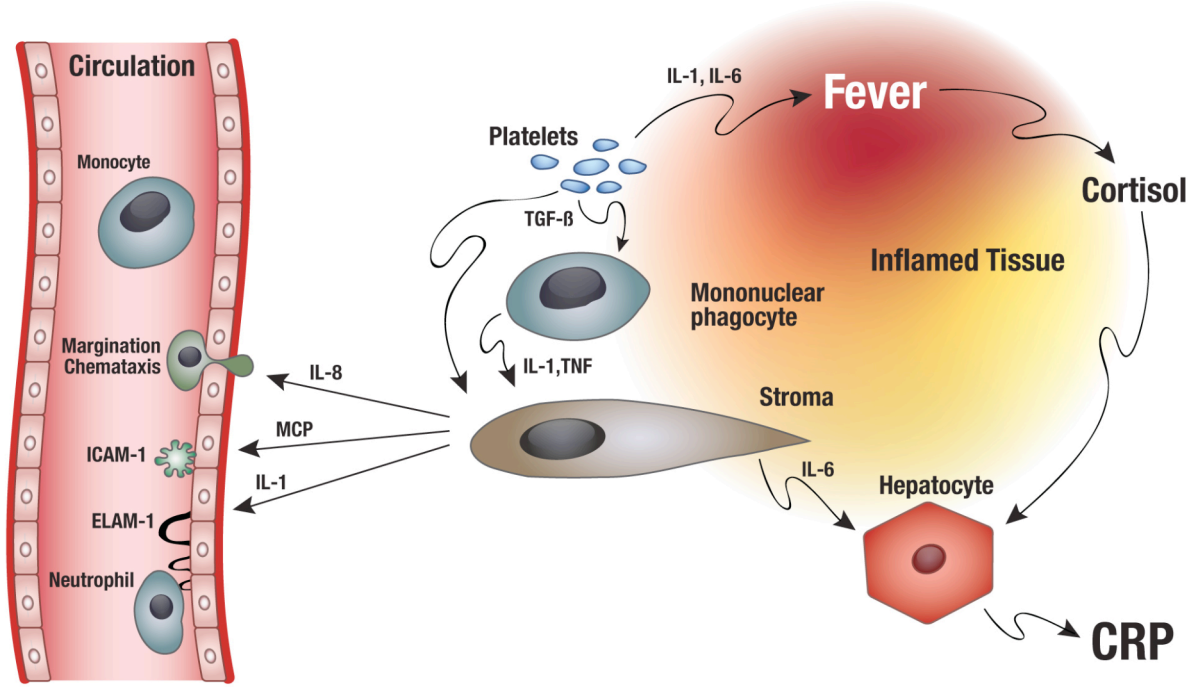


Figure 2. A basic overview of the cell and cytokine interaction during the acute phase response. Platelets and mononuclear phagocytes release early cytokines, (IL-1 and TNF) at the site of tissue damage. These will active adjacent cells to produce other chemotactic cytokines and initiate the accumulation of inflammatory cells. The hepatic response is also activated by these cytokines, and these will activate acute phase proteins, such as CRP.

C-reactive protein

CRP is considered the prototypical acute phase protein in humans. It rises dramatically in response to an infection or inflammation, but also slightly increased levels of CRP have been linked to disease, e.g. it has been suggested to be an indicator of atherosclerotic vascular diseases. CRP is synthesized in the liver in response to interleukin -6 and IL-1β, and its pentameric structure allows an efficient binding of both the ligands and receptors on effector cells (fig. 3.). A recent study revealed that also IL-17A could stimulate CRP expression, and this was independently of IL-1β and TNF [20].

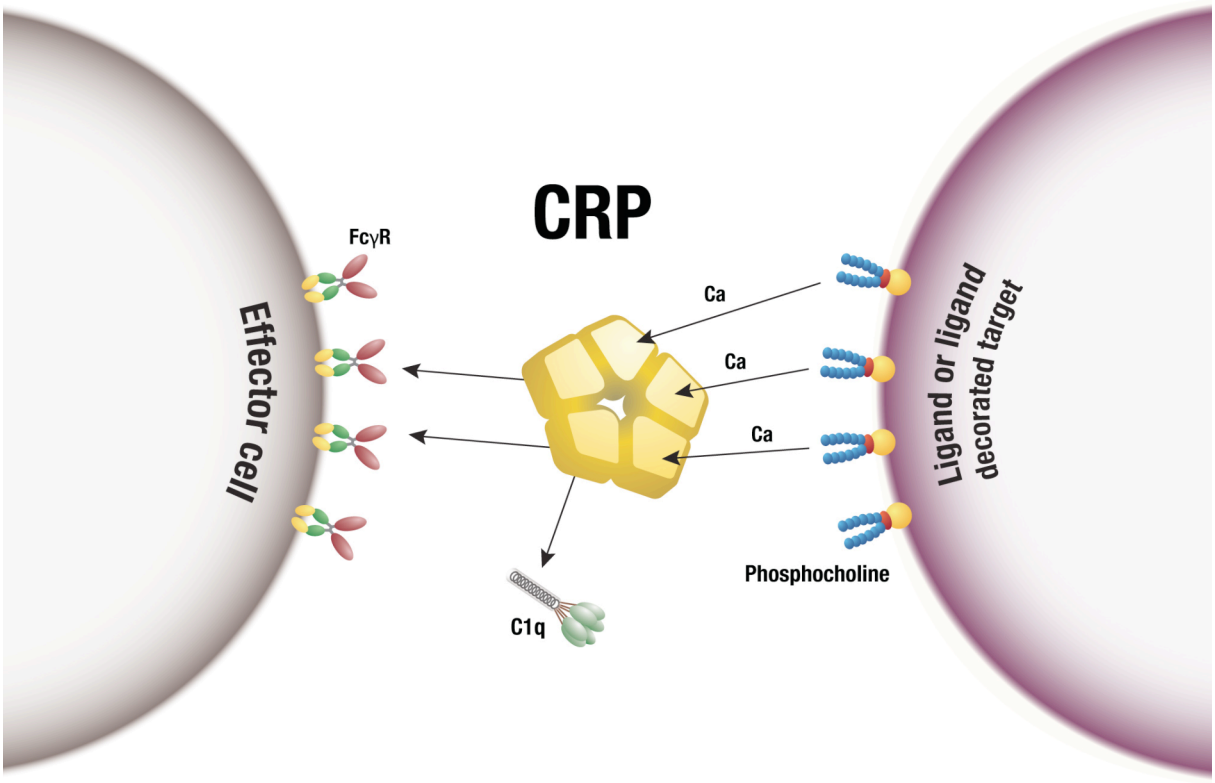


Figure 3. The pentameric structure of CRP facilitates an efficient binding of both ligands, here represented by phosphocholine, and receptors on the effector cells or complement proteins.

The best-characterized ligand for CRP is phosphocholine (PC), and this is responsible for the binding of CRP to several microorganisms, including the C-polysaccharide of pneumococcus [21], the phosphorylated disaccharide on *Leishmania donovani* [22], the phosphorylcholine-expressing *Neisseria meningitides* [23], and the LPS of *Hemophilus influenzae* [24]. CRP can also bind to apoptotic and necrotic cells, promoting the early classical complement pathway, although not efficiently generating the membrane attack complex, due to interaction with the complement regulatory protein, factor H. Activation of the classical complement cascade, has been shown to contribute to the killing of *Schistosoma pneumoniae* in mice [25]. The opsonization and phagocytosis of apoptotic cells by macrophages is improved by the binding of CRP, this also promotes an anti-inflammatory response due to the increased production of IL-10 (fig. 4) [26].

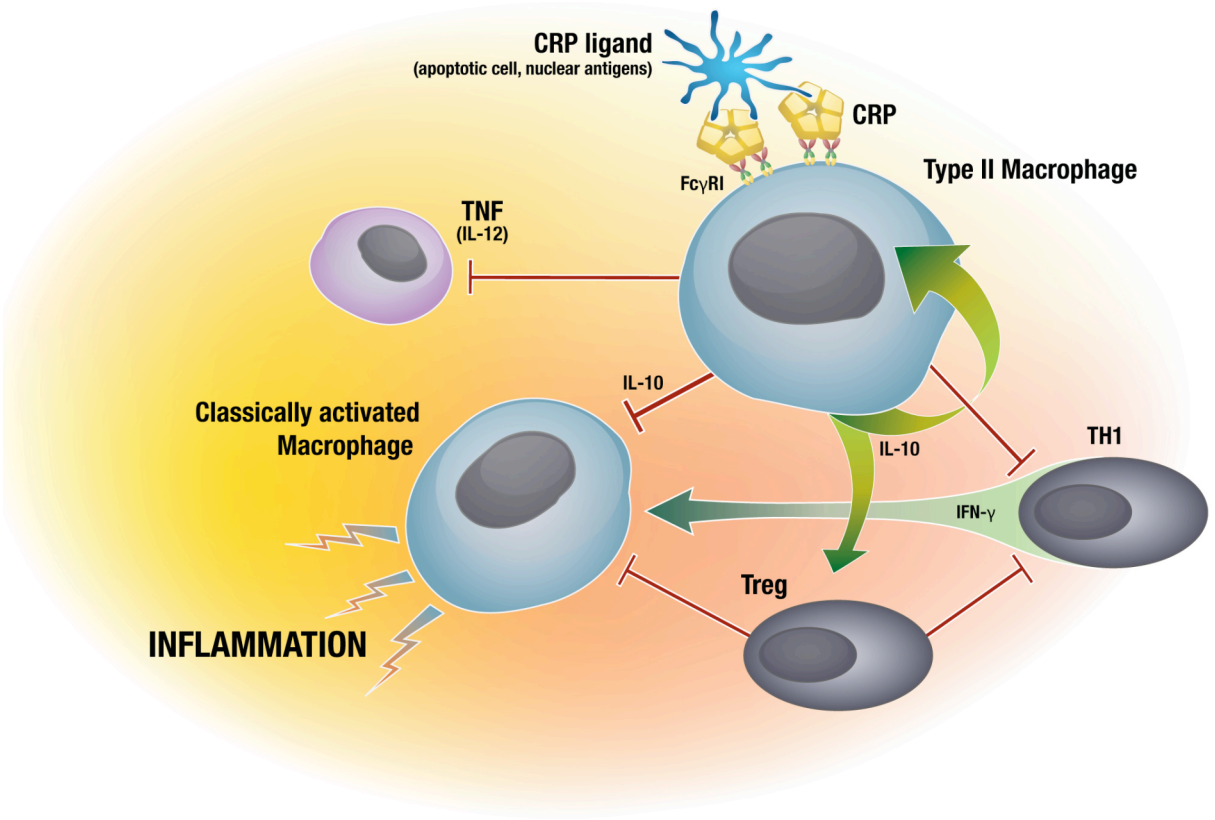


Figure 4. A possible mechanism of the inflammatory properties of CRP. The binding of CRP to macrophages induces the production of IL-10. This turns off the production of IL-12 and TNF, and down-regulates Th1 cells and classically activated macrophages, and activates regulatory T cells, which further limits the inflammation. Hence, the net-effect of CRP in anti-inflammatory.

Binding of CRP to the FcγR increases the phagocytic capacity of the effector cells, and the affinity of CRP to FcγRI actually exceeds the affinity of IgG. Although the overall structure of CRP and immunoglobulin is quite diverse, there is a sequence homology between the Fcγ binding sites on IgG and CRP [27]. This could be a reason for the IgG-like biological effects of CRP when it binds to FcγR. The binding to FcγRIIa appears to be allele specific; CRP binds well to one of the allelic variants, while IgG2 binds well to the other. Interestingly, so far there is no evidence for a binding of CRP to FcγRIII, the most pro-inflammatory FcγR, which is contradictory to the pro-inflammatory label given to CRP.

CRP has shown direct effects on DC differentiation, maturation and function [28], and neutrophil chemotaxis and signalling [29], while other effects of CRP on cells of the immune system are more indirect, for instance by stimulating differences in the cytokine milieu. CRP has also showed a capacity to induce a tumoricidal activity by murine macrophages [30].

The circulating levels of CRP has been show to be associated with socioeconomic status in several high-income countries, moreover, the levels of circulating CRP were higher in

individuals of African, Latin or South Asian descent as compared to those of European descent [31]. The significance of these minor elevations have been discussed mainly in atherosclerosis, but it is now clear that minor differences in CRP concentrations may have an effect on many other conditions. In malaria, these two observations could be of importance, since individuals living in areas with malaria most often are of non-European origin, and are often living in developing countries. It may be suggested that these minor elevations in CRP levels can be involved in the protection against malaria. However, whether elevated CRP levels are beneficial or not in relation to malaria, still have to be clarified.

Adaptive immune cells

B cells are considered to be the primary player in humoral immunity, and they are the only cells capable of producing antibodies, each B cell producing antibodies of a unique specificity. For some antigens, the B cells require co-stimulatory signals from Th cells, these antigens are called thymus dependent (TD) antigens. The thymus independent (TI) antigens do not need the help of Th cells to be processed. Common TI antigens are polysaccharides, LPS, peptidoglycan and lipoprotein, while TD antigens often are proteins. In response to TD antigens, germinal centres are being formed. Here three important B cell differentiation events take place: affinity maturation, class switching, and formation of plasma cells or memory cells. Germinal centres are required for the affinity maturation and memory B cell formation, while some class switching and many plasma cells can be formed outside these centres.

Regulatory B cells have been described in experimental models of autoimmunity, infections and cancer. Their regulatory function appears to be mediated by the production of IL-10, however it seems as the differentiation of regulatory B cells is not affected by the Th1-Th2 microenvironment [32]. So far, regulatory B cells have only been described in mouse studies, but there is data from humans suggesting a possible regulatory B cell subset. A reduced proportion of IL-10 producing B cells have been reported in multiple sclerosis (MS) patients that could suggest a reduced regulation by B cells [32]. Experimental parasitic models have revealed that neutralizing antibodies and/or anti-inflammatory mediators from Fcγ positive cells are involved in mediating the B cell regulation [32].

T cells are divided into αβ T cells and γδ T cells, depending on the composition of the T cell receptor (TCR). The αβ T cells are further divided into CD4+ T cells, which regulate the

cellular and humoral immune responses, and CD8+ T cells, that show a major cytotoxic activity toward cells infected with intracellular pathogens. Both CD4+ and CD8+ T cells can develop into memory T cells, which further increases the specificity of the adaptive immune responses.

The CD4+ T cells are further divided into **Th1/Th2/Th17** types of cells depending on the cytokine profiles they produce (fig. 5). T helper cells are involved in organizing the immune response against several pathogens and antigens, i.e. the previous mentioned TD antigens (discussed more in detail in the B cell section). Th1 cells produce IFN-γ and drive the type-1 pathway, the cellular immunity pathway, to fight viruses and other types of intracellular pathogens. The Th2 cells produce IL-4, IL-13 and IL-25 and are said to drive the type-2 pathway, the humoral immunity pathway, by up-regulating antibody production to fight extra-cellular pathogens [33]. Th17 cells were described quite recently, and have now been accepted as an independent Th cell subset, with major functions in the host protection against extracellular pathogens that have escaped the Th1-Th2 type of responses. The key cytokines produced by Th17 cells are IL-17A, IL-17F and IL-22 [34].

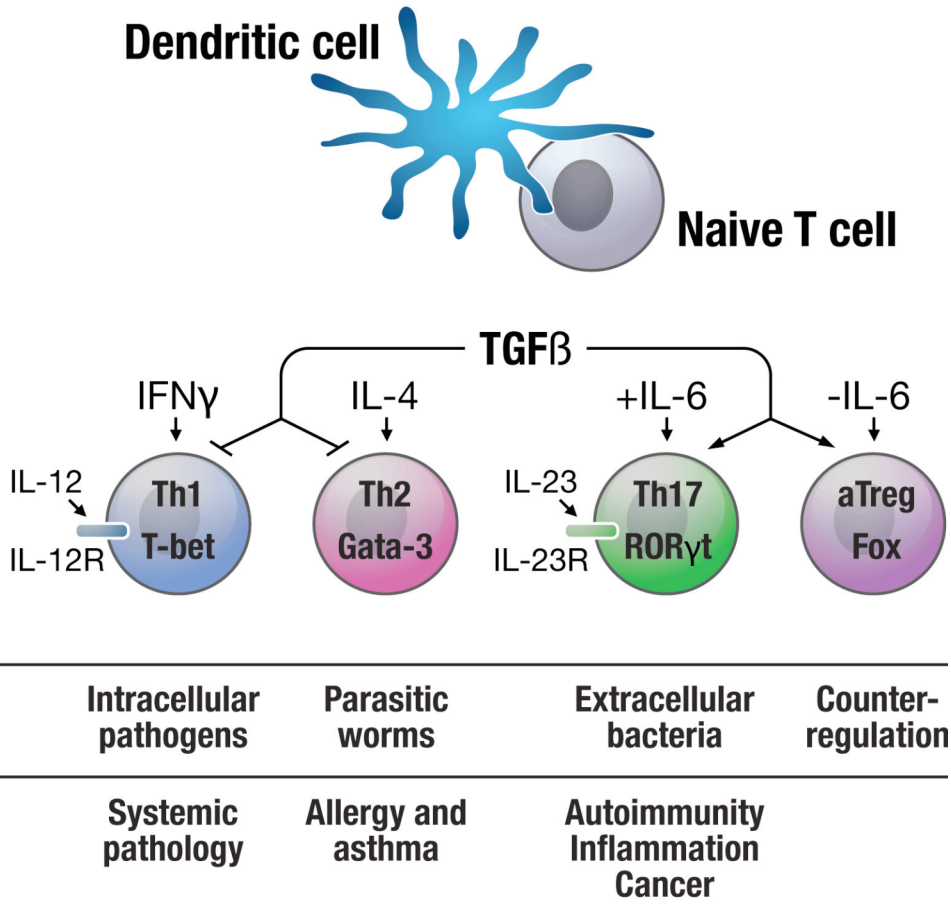


Figure 5. CD4+ T cell differentiation can lead to different subsets of Th cells or Tregs depending the initial stimulus [35], these subsets of T cells have different functions and cytokine production profiles.

A less characterised subset of T cells is the **T-regulatory cells** (Treg), which can regulate the responses by CD4+ and CD8+ T cells and NK cells. Three main subsets of CD4+ Tregs have been described; the CD4+CD25+ Tregs are naturally occurring at a frequency of 5-10% of the total peripheral CD4+ T cells, and the two induced Treg subsets, the CD4+ Treg type 1 (Tr1), and T helper 3 regulatory T cells (Th3), which are classified based on the cytokines they produce [35]. Tr1 cells produce high levels of IL-10 and Th3 cells produce high levels of TGF-β. All Tregs are depending on TCR triggering to obtain their suppressive function. However their suppressive activity seems to be antigen-nonspecific once the Treg have been activated [35]. Naturally occurring Treg have a cell-cell contact required suppression, while Tr1 and Th3 exert their suppressive function by the production of soluble immunosuppressive cytokines.

NK T (NKT) cells express a αβ-TCR, and are therefore by definition T cells, however they share some of the characteristic NK cell markers. At least two subsets of NKT cells are distinguishable, CD4+ and CD4-, where some can be CD8+. The NKT cells can be found wherever T cells are found. In contrast to other T cells, classical NKT cells do not interact with MHC class I, or II, but do interact with glycolipids presented by CD1d, a non-classical antigen-presenting molecule. They can also up- or down regulate immune responses by secretion of Th1-, Th2- or regulatory cytokines [36].

The γδ T cells, representing a relatively small part of the T cell repertoire, recognise non-peptidic antigens in a MHC independent manner [37]. γδ T cells are believed to belong somewhere in between, not being completely adaptive or innate. However, recent studies do suggest a more innate action of these cells, albeit probably the most complex and advanced cellular representative of the innate immune system. Activated γδ T cells have shown a professional antigen presenting function [38], and there is also evidence for γδ T cells and DC to exert a regulatory influence on each other.

Antigen presentation

Key functions for the immune responses are the antigen recognition and presentation, the antibodies produced by B cells can bind directly to the naïve antigen, but T cells usually need to get the antigen presented as a peptide bound to a MHC molecule. Two classes of MHC molecules function in antigen presentation, MHC class I and MHC class II. The MHC class I molecules are expressed on almost all cells, they primarily present endogenous antigens (e.g. viral proteins), and it is mainly CD8+ T cells that recognise the MHC class I, leading to lysis

of cells presenting foreign peptides [39]. The MHC class II is only expressed on the professional APCs, which traditionally comprise B-cells, macrophages and DC. They express the MHC class II molecules together with co-stimulatory molecules that are necessary for a proper T cell response. MHC class II presents peptides from exogenous proteins, and the APCs present almost exclusively to CD4+ T cells [39].

Immunoglobulins

The immunoglobulin (Ig) molecule can be found both as membrane bound on B-cells and in a secreted form, that is produced by plasma B-cells. When bound on the cell surface, the Ig functions as a receptor involved in differentiation, activation and apoptosis, while the secreted form can neutralize foreign antigens and recruit other effector components [40]. The Ig consists of two large polypeptide chains, called heavy chains, and two shorter, called light chains, paired together in a Y-shape (fig. 6). The open upper part of the Y is the antigen binding part, called Fragment antigen binding (Fab), and the lower part of the Y is the fragment crystallisable (Fc) part, which is responsible for interaction with receptors and complement [40]. There are five different Fc parts, each corresponding to an Ig isotype, IgM, IgD, IgG, IgA and IgE (fig. 6), all of which can function both as receptors on the cell surface and in a secreted form [40].

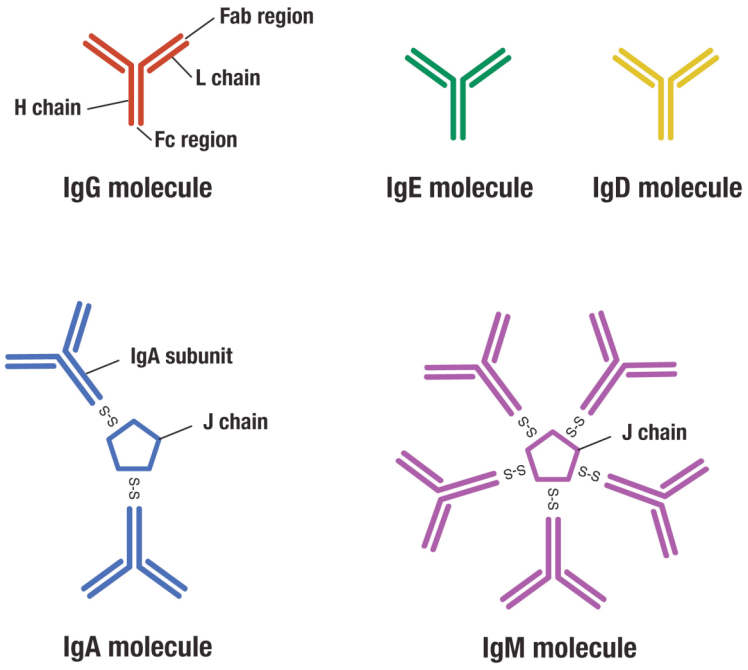


Figure 6. The basic structure of the Ig molecule, and the five different Ig isotypes. The J chain in the pentameric form of IgM and the polymeric IgA are believed to enable secretion of the antibodies into mucosal tissues.

IgM is the first antibody class that encounters a new antigen [41], and it can be found in two forms; membrane bound monomeric IgM and secreted pentameric IgM. The pentameric form of IgM makes it a powerful complement activator and it can up-regulate both primary and memory responses and increase affinity maturation [42]. It is the first antibody to be expressed in the fetus, however IgM does not cross the placenta.

Out of the total serum immunoglobulins, IgD represents only a small fraction. It appears not to cross the placenta, and it has a weak or absent binding to normal lymphocytes, neutrophils and monocytes [43]. Native IgD has little or no capacity to activate complement effects, whereas aggregated monoclonal IgD induces complement activation [43]. IgD is co-expressed with IgM on most peripheral B-cells. IgD is conserved across different species and it is found in all mammalian and avian species, suggesting an evolutionary advantage [43]. The role of IgD is still not completely understood, but it seems to behave like IgM early in infections. Moreover, IgD concentrations are elevated in chronic infections, but if these specific IgD antibodies are of any clinical importance is unknown [43].

IgG is dominating the humoral immune responses in humans. Human IgG is divided into four subclasses, IgG1-IgG4, where IgG1 is the largest subclass (66%), followed by IgG2 (24%), IgG3 (7%) and IgG4 (3%) [44], however this may vary between responses against different pathogens. IgG2 is the main antibody targeting encapsulated bacteria, IgG1 and 3 are mainly directed against protein antigens and IgG4 is common in chronic exposure to protein antigens and in allergy. The IgG subclasses also differ in their ability to activate complement, IgG3 and IgG1 being the most effective ones, and IgG2 is a weak activator, whereas IgG4 does not activate complement at all. IgG is the only antibody that can pass the placenta, providing the foetus with protection from pathogens capable of crossing the placenta.

IgA is the most abundant antibody in secretions [45]. Mucosal surfaces are the main source of antigenic material in the body, and in the mucosal tissues, the local synthesis of secretory IgA is dominating over that of the other antibody classes. Secretory IgA is present on all body surfaces except the skin, and is therefore an important first line of defence, and it can trigger cell-mediated events [45]. The transport of the polymeric IgA to the mucosal surface is enabled by the poly-Ig receptor that will bind the poly-IgA and transport it through the cells. This transport is very important for the child, since IgA is the major immunoglobulin in breast milk and colostrum, providing the child protection against intestinal pathogens [46]. Maternal

IgA and IgG, from the placental transport, support the neonate's immune system during the first period of life.

IgE is the antibody class that is the least abundant in human serum, however it is very capable of triggering many powerful immune reactions. The effect of IgE is mainly known in allergy, where IgE mediates the hypersensitivity reactions responsible for the symptoms of hay fever, asthma, hives, and anaphylactic shock. IgE can up-regulate carrier-specific antibody responses [42], both primary and memory responses [47]. Elevated levels of IgE have been shown for many helmintic infections [48-50] and also in malaria exposed individuals [51-54].

Fc receptors

One receptor type that is involved in antibody recognition comprises the FcR. They recognize and bind to the Fc part of all Ig classes, FcγR recognize and bind IgG, FcαR for IgA, FcδR for IgD, FcμR for IgM and FcεR for IgE. Signalling through the FcR induce many different actions, depending on the cell carrying the receptor, e.g. phagocytosis and release of inflammatory components [55]. There are also FcR responsible for transportation of Ig through epithelia; they are the polymeric IgA and IgM receptors and the neonatal FcR, which mediates antibody transportation through the placenta from the mother to the child [55]. The FcR capable of cell activation all contain intracytoplasmic activation motifs, designated immunoreceptor tyrosine-based activation or inhibiting motifs (ITAM or ITIM) [56]. These ITAM can be of two different types, multi-chain or single-chain receptors. The FcR that lack ITAM do not trigger cell activation, the exception is FcγRIIIB, which has no activating effect on its own, but contributes to cell signalling by associating to other FcR [55].

In humans, there are three families of FcR binding IgG, FcγRI (CD64), -RII (CD32) and -RIII (CD16). FcγRI is a high-affinity receptor that binds monomeric IgG, FcγRII and -RIII are low-affinity receptors only binding complexed or aggregated IgG. These receptors are either activating (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, FcγRIIIb) or inhibitory (FcγRIIb), and these receptors are expressed throughout the haematopoietic system. Only NK cells and B cells do not co-express activating and inhibiting FcγR: NK cells only express activating FcγR, while B cells only express the inhibitory FcγRIIb [57]. Follicular DC, endothelial cells, microglial cells, osteoclasts and mesangial cells also express FcγR [57]. FcγR have well defined roles in the triggering of innate cell functions, resulting in the release of cytokines and chemo-

attractants, and an increased phagocytic capacity of effector cells [55]. Moreover, FcγR also function in antigen presentation and immune-complex mediated maturation of DC, and in the regulation of B-cell activation and plasma-cell survival (fig. 7) [57]. The multitude of innate and adaptive immune reactions that involve FcγR make them interesting in many different diseases and also make them attractive targets for new immunotherapeutic designs [58].

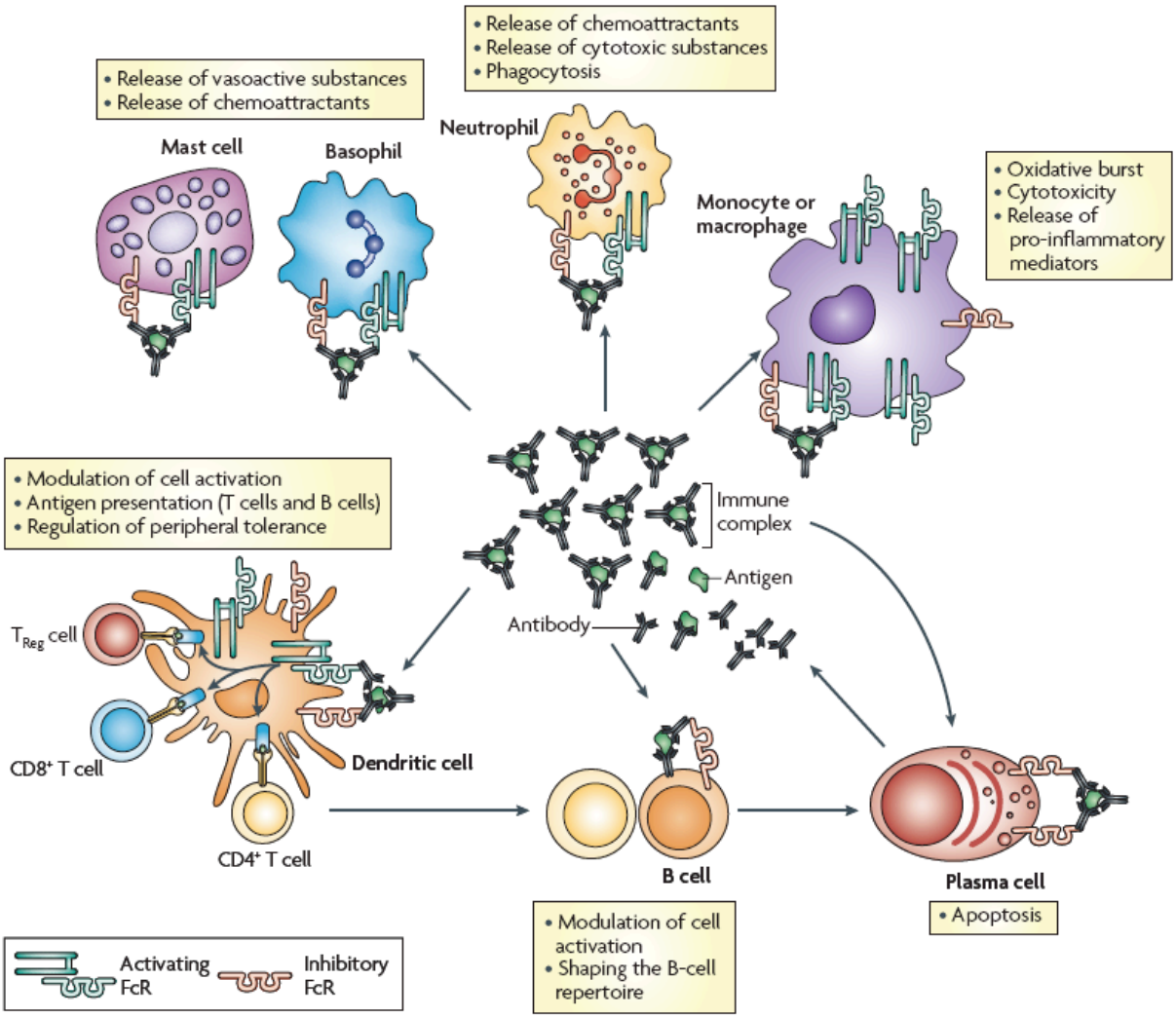


Figure 7. Regulation of immune complexes through FcR. All cells expressing FcR can be activated or inhibited in their function by binding of immune complexes to the FcR, leading to the release of effector molecules or other cell functions. Adapted from Nimmerjahn *et al* [57].

Cytokines

Cytokines are proteins or glycoproteins with a regulatory capacity, secreted by cells in response to stimuli. In addition to assisting in development of various immune cells, they can also regulate the intensity and duration of the immune response and induce the inflammatory response. The action of cytokines is through specific receptors on the cell surface, binding to these receptors triggering signal transduction pathways that will alter the gene expression of

the target cell, and hence alter the function. Most cytokines have a local effect, with either an autocrine (binding to receptors on the same cell that secreted it) or paracrine action (they are binding to a nearby cell). Very few cytokines can exert an endocrine effect, this requires that the cytokine is released in the circulation and transported to distant parts of the body.

Cytokines rarely work alone; it is more common that effector cells are influenced by a certain cytokine milieu, containing several cytokines that enhance or counteract each other in an intrinsic manner. Th1/Th2 type of cytokines is a rough division trying to simplify the different effects cytokines could have on the cells. The main representatives of the Th1 types are IFN-γ, IL-2 and IL-12, and this is a response mainly raised against intracellular pathogens. Extracellular pathogens activate the Th2 types of cytokines, with IL-4 as the most important one.

Up till now, more than 100 cytokines have been described, and it has been estimated that more than 300 different ones exist. The following section is therefore only considering the cytokines that are of particular interest for the work presented in this thesis.

Interleukin 1β

IL-1β is a pro-inflammatory cytokine, which can activate inflammatory cascades, including a self-amplifying effect. IL-1β induces many different genes and suppresses others, giving rise to a variety of biological effects [59]. IL-1β is an important fever molecule, a potent acute phase inducer, and is also implicated in the pathogenesis of several diseases. Some of the variation in CRP levels has been associated to gene polymorphisms in the IL-1β gene. A recent study showed that three gene polymorphisms in the promoter region predict clinical levels of both IL-1β and CRP [60].

Interleukin 6

IL-6 has a wide variety in its functions; it can induce antibody production by B cells, induce the acute phase response, induce T cell growth and differentiation of cytotoxic T cells among many other biological functions [61]. The biological functions exerted by IL-6 act through receptor complexes that can be either membrane bound or soluble [62]. The soluble receptor can prolong the half-life of IL-6 in plasma, and the importance of this receptor has been shown in many different clinical conditions, i.e. inflammatory disorders, neurological conditions, cancers, HIV and malaria [63].

Interleukin 10

IL-10 is an anti-inflammatory cytokine, capable of inhibiting pro-inflammatory cytokines and chemokines, including IL-1β, IL-6 and TNF. Moreover, IL-10 can inhibit NK cell and macrophage activity, but also stimulate B cell activity and T cell development. IL-10 is a very important player in the clearance of intracellular pathogens; higher IL-10 gives a faster clearance, but often with a more severe pathology [64]. CRP can induce IL-10 [26], and IL-10 has been shown to regulate the expression of FcR via signals through TLR4 [65]. IL-10 is also involved in the generation of peripheral Tregs [66]. Functional polymorphisms in the promoter region of the IL-10 gene have been associated with the expressed levels of this cytokine [67, 68].

Tumour necrosis factor

TNF is strongly regulating the inflammatory response, and it has been implicated in many inflammatory, infectious and malignant conditions [69]. The main source of TNF is activated macrophages and monocytes, but also other cells can produce TNF, including NK cells, B and T cells, mast cells, neutrophils, endothelial cells and fibroblasts [69]. TNF is also a strong inducer of the acute phase response, and is also very important in the host defence to bacterial, viral and parasitic infections.

Interferon-γ

IFN-γ is the key Th1 cytokine, and it exerts its pro-inflammatory effect mainly through macrophages [70]. IFN-γ can limit the parasitemia in the early stages of the malaria infections [71], and it has been associated with resistance to infections [72].

Malaria

History of malaria

The earliest finding of a *Plasmodium* parasite is from a mosquito preserved in amber from the tertiary period [73]. Throughout the history of mankind, malaria parasites have evolved together with us [74], and they have had a tremendous effect on our genome, with a selection for malaria protective, but otherwise harmful genetic variations, such as sickle-cell anaemia, thalassemias, and glucose-6-phosphate dehydrogenase (G6PD) deficiencies. Due to the characteristic cyclic fevers in malaria, references to malaria can be found in writings from the Sumerians from Mesopotamia, the Chinese Nei Ching (2700 BC), the Indian Vedic writings (1600 BC) and the papyri from Egypt [75]. The person who got the credit for describing malaria is however Hippocrates (460-370 BC), a Greek physician, who was the first person to describe the periodically occuring fevers together with splenomegaly and connected these fevers with pregnancy complications [75]. The main theory regarding the cause to the periodical fevers was suggested to be the bad air (Italian: mal aria) coming from the wetlands, hence the name malaria. This was still the general belief until Giovanni Maria Lancisi (1716) discovered that draining of swamps limited malaria, and he suggested an insect as the origin of the disease. His idea was however not proven until Ronald Ross in 1897 observed malaria parasites in an anopheline mosquito gut. The French army surgeon Charles Louis Laveran discovered the malaria parasite in 1880, and Camillo Golgi identified additional malaria species in 1885.

Already the Egyptians used bednets, and the wormwood derived artemisinine treatment has been used to treat fevers in China since 168 BC. Quinine is a product from the bark of the *Chinchona* tree, that was used to treat the periodically cyclic fevers in South America. The first widely distributed and used drug against malaria, chloroquine, was discovered by the chemist Hans Andersag in Germany in 1934, but not until 1946 was this drug recognized as a safe and effective anti-malarial agent. Together with DDT, chloroquine was distributed around the world and the eradication of malaria was predicted to be easy, but only a few decades later, parasite and mosquito resistance were reported. Up till this day, scientists are working on finding new effective means to control malaria. Recently, the ancient Chinese remedy artemisinine was re-discovered as an effective treatment, especially in combination

with other anti-malarials, and this combination therapy is now the first line treatment to use in many countries [76].

Prevalence, severity and impact on world health

Malaria is the most prevalent infectious disease in the world (fig. 8), causing approximately 247 million acute clinical cases and 880.000 deaths every year (WHO report from 2006). About 90% of the deaths related to malaria occur in sub-Saharan Africa, and the disease is the leading cause of mortality (20%) in children less than five years of age (WHO. Roll Back Malaria). Women are also highly susceptible to so called placental malaria (or pregnancy associated malaria, PAM) during their first and second pregnancy, which may lead to death. Moreover, malaria infections in the mother can lead to spontaneous abortion, neonatal death and low birth weight of the child.

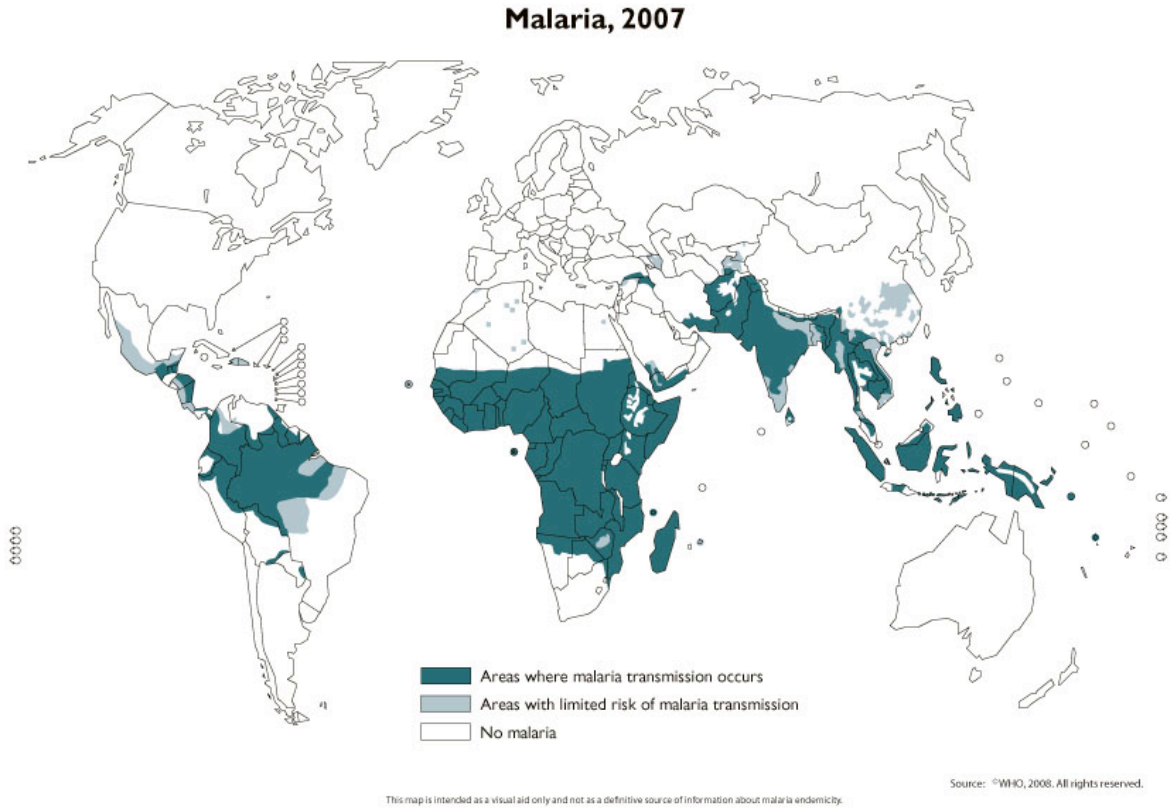


Figure 8. Malaria distribution 2007 (www.who.int/ith/maps/malaria2007.jpg). Printed with permission from WHO.

Today, malaria is a global problem. Natural disasters, agricultural projects, climate changes and the increased interest of travels to countries with endemic malaria, are giving the parasite and the mosquito many chances to spread to previously non-malaria areas, or to regain areas where it had been eradicated. The rapid development of drug-resistance amongst the malaria

parasites is another problem, since several of the anti-malarial drugs available are now loosing their effect, and the alternative drugs are expensive. An optimistic report from Malawi showed that the previously widely spread chloroquine resistance among the *Plasmodium falciparum* parasites in this country, seems to have disappeared, and chloroquine is again an effective anti-malarial drug [77]. The vector has also been a target for control measurements of the disease, the major ones being insecticide usage on wetlands and insecticide treated bed nets. However, the mosquitoes have developed resistance to many of the insecticides, leaving the insecticide treated bed nets as the only effective barrier. The development of a functional vaccine has been a main goal for controlling malaria infections around the world, but the progress has been slowed down by the limited knowledge of how a protective immunity to malaria is developed and what protective components that are involved in parasite neutralization.

Malaria parasites and life cycle

Malaria is caused by a protozoan parasite of the *Plasmodium* family. Although five species have been shown to infect humans, *P. falciparum, P. vivax, P. malariae, P. ovale* and recently *P. knowlesi*, only *P. falciparum* results in high mortality as a result of its prevalence, virulence and drug resistance [78]. However, the few cases reported with *P. knowlesi* malaria have also showed a high mortality, really making it important to regard this parasite as a new serious life-threatening human malaria parasite [79].

Transmitted by the female *Anopheles* mosquito, the sporozoites reach the liver, where they develop into merozoites. After 1-2 weeks, the infected liver cells rupture, releasing thousands of merozoites, which all can invade red blood cells (RBC). *P. vivax* and *P. ovale* have a special feature in that some sporozoites of these two parasites do not immediately develop into merozoites, but instead produce hypnozoites. These remain dormant for periods ranging from weeks up to several months or years, and after the period of dormancy, they reactivate and produce merozoites. Hypnozoites are responsible for the long incubation and late relapses in these two species of malaria [80]. Inside the RBC the parasite develops into the asexual blood stages, called rings, trophozoites and schizonts. When the schizonts rupture, many merozoites are released, which invade new RBC, initiating the erythrocytic life cycle of the parasite (fig. 9). Some merozoites develop into gametocytes, which are taken up by the anopheline mosquito, and the parasite starts its sexual cycle inside the mosquito midgut,

developing into zygotes, ookinetes, oocysts and finally into sporozoites, that migrate to the salivary glands [78].

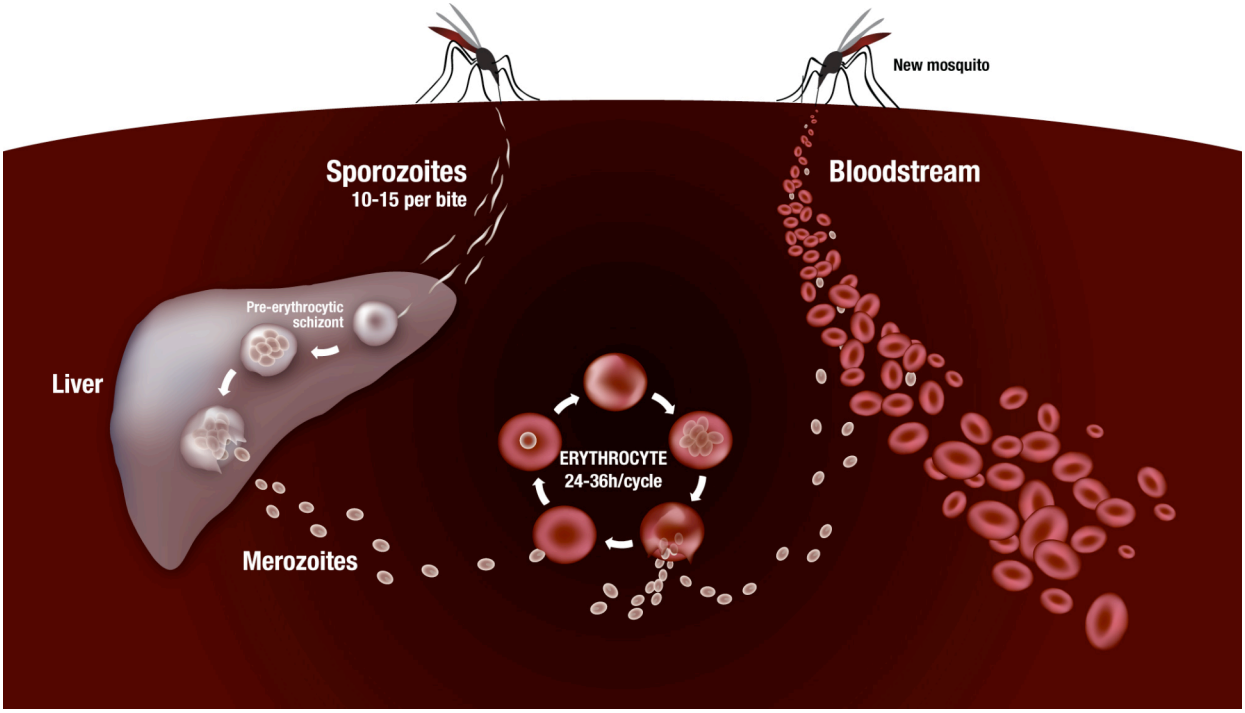


Figure 9. The asexual life cycle of the *Plasmodium* parasite.

The erythrocytic cycle of *P. falciparum* has a unique feature, mature trophozoites and schizonts are sequestered in the peripheral circulation, due to parasite mediated changes of the surface of the infected RBC (iRBC), causing them to adhere to endothelial cells (sequestration), other un-infected erythrocytes (rosetting), and to platelets (clumping) (fig. 10). It is an accepted theory that this adhesion is an immune escape mechanism of the parasite, and that it also may lead to a better maturation in the microaerophilic venous atmosphere [81].

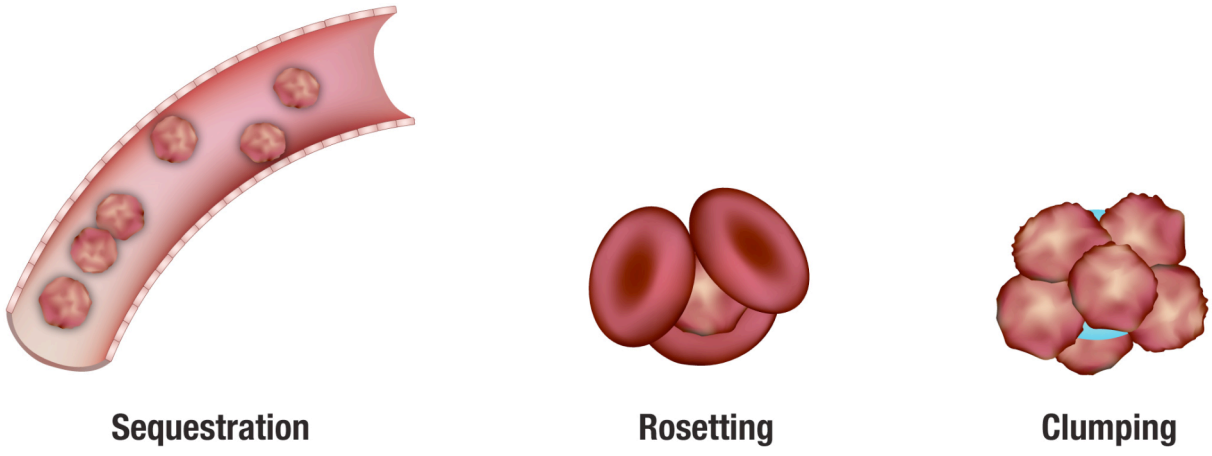


Figure 10. Illustrative figures of sequestration, adherence to endothelial cells, rosetting, adherence to un-infected erythrocytes, and clumping, adherence of infected erythrocytes to platelets.

Malaria disease

The clinical symptoms of malaria are only presented during the erythrocytic life cycle of the parasites, and mild or uncomplicated malaria is characterized by fever, followed by nausea, headache, cough, diarrhoea and muscular pain. Infections by *P. vivax, P. ovale* and *P. malariae* usually give milder malaria as compared to those caused by *P. falciparum*. This is due to the ability of *P. falciparum* to adhere to host endothelium in combination with the high parasitemia usually seen for *P. falciparum*. The WHO defines severe malaria as a parasitemic person with one or more of the following symptoms: prostration (inability to sit up without help), impaired consciousness, respiratory distress or pulmonary edema, seizures, circulatory collapse, abnormal bleeding, jaundice, hemoglobinuria or severe anaemia (haemoglobin < 50 g/L or hematocrit < 15%) [78]. Cerebral malaria is caused by the sequestration of *P. falciparum* infected RBCs in small blood vessels in the brain, causing blocking of the blood flow, leading to coma or other neurological phenomena, such as seizures and elevated intracranial pressure.

Malaria Immunology

Naturally acquired immunity to malaria

A proper immune response against malaria

The details in the **pre-erythrocytic immune responses** in humans are still poorly defined. However, it is believed that both antibody-mediated as well as cellular mechanisms have important roles. Antibodies have been found to block the initial sporozoite invasion of hepatocytes. Antibodies have also been shown to kill sporozoites directly by opsonization and to recognize parasite-derived proteins on the surface of infected hepatocytes, and thereby promote killing of the infected hepatocyte [82]. Both CD4+ and CD8+ cytotoxic T cells can directly eliminate infected hepatocytes, and NK and NKT cells have shown to provide help for the activation, differentiation and the effector activity by T and B cells [82].

The immune response to the **erythrocytic stages** is primarily conferred by humoral components, due to the absence of MHC molecules on red blood cells. However, monocytes [83], macrophages [84] and NK cells [85] can kill the parasite in the absence of antibodies, probably involving binding of CD36 to parasite derived surface molecules on iRBC [86]. Human NK cells have also been shown to rapidly produce IFN-γ, a cytokine associated with reduced susceptibility to malaria [72, 87], suggesting an importance of NK cells early in the blood-stage malaria infections [88]. The NKT cells produce large amounts of IFN-γ and IL-4, when activated through the TCR, and this rapid cytokine output may activate other lymphoid cells [89]. The CD1-restricted NKT cells can also contribute to malarial splenomegaly, in response to a *P. berghei* infection, which is associated with expansion of splenic B-cells and enhanced parasite-specific antibody formation [89]. The role of DC in malaria immunity is still relatively unknown, some studies show that the maturation of human DC is suppressed, and that their ability to activate T-cells is reduced by iRBC [90, 91]. However, using animal models, it was demonstrated that DC from infected mice are fully functional APC [92]. It has been shown that depending on what parasite strain that is infecting, the DC respond differently. The DC in mice infected with a lethal strain of *P. yoelii* were non-functional, while in mice infected with a non-lethal strain, the DC were functional and capable of activating T cells and secret cytokines [93]. This could be a reason for the different results obtained in the different studies.

During the first few days in a malaria infection, γδ T cells expand and they have been shown to have the capacity to directly inhibit the parasite growth [94]. Both the CD4+ and CD8+ T cells play important roles in immunity to malaria, but at different stages. During the liver stage CD8+ T cell functions are important [95], and they also contribute to protection against severe malaria [96, 97]. The CD4+ T cells are crucial in the immunity against asexual blood stage malaria. They produce cytokines that are involved in the activation of innate immune responses, and they are also required for the B cell production of anti-malarial antibodies. The immunity to blood stage malaria is dependent on the CD4+ T cells, anti-malarial antibodies and B cells [98]. For other protozoan infections, the Th1/Th2 balance is crucial for the clearance of the parasites [99]. In malaria, many studies, using mouse models, have shown that pro-inflammatory Th1 cytokines are crucial determinants of the outcome of the malaria disease. C57BL/6 mice, which have a predominant Th1- immune response during a malaria infection, are more susceptible to cerebral complications, than the Th2 biased BALB/c mice, which are resistant to cerebral malaria [100]. In humans, studies have shown a possible association between IL-4 and levels of anti-malarial antibodies [101]. Tregs are still under investigation for their role in malaria immunity [102]. The role of TLR is still not fully understood, but it is known that they can recognise malaria parasites or their metabolites. The glycosylphosphatidylinositol (GPI) anchors of *P. falciparum* have been shown to mediate signals, mainly through TLR2 and to a lesser extent by TLR4 [103]. Furthermore, was hemozoin, a parasite heme metabolite, reported to be recognised by TLR9 [104], but this binding appears rather be due to parasite DNA associated with the hemozoin [105]. Common polymorphisms in TLR4 may be associated to the clinical outcome of a malaria infection [106], and polymorphisms in TLR4 and TLR9 have been shown to increase the risk of low birth weight in *P. falciparum* infected pregnant women and the risk of maternal anaemia [107]. However, none of these polymorphisms were found to affect the parasite prevalence or density of the *P. falciparum* infection [107].

Development and duration

Unlike many other acute infections, malaria does not induce a long lasting immunological memory; it rather develops gradually and requires repeated infections to persist. The developed immunity is not sterile; malaria parasites can still survive in the host, but at such low levels that the clinical symptoms do not appear. The development of this semi-immunity is influenced by age, genetic background, pregnancy, co-infections and the nutritional status of the host. Artavanis-Tsakonas *et al* [88] have proposed a simplified model, suggesting that

the primary infection in infants living in endemic areas will induce minimal clinical symptoms due to the low induction of IFN-γ and TNF by an innate pathway. Naive T cells will be primed, and during the next encounter these antigen-specific T cells will produce high levels of IFN-γ and up-regulate the production of TNF by macrophages and other cells. These high levels of pro-inflammatory mediators will lead to an increased risk of severe manifestations, such as cerebral malaria or systemic shock. During the subsequent exposures, the infection will be controlled by an effective anti-malarial immune response that has matured since the previous infections, and this will lower the antigen levels. The lower antigen load will shift the immune response from a pro-inflammatory to a regulatory response, which will limit the dangerous overproduction of inflammatory mediators. Individuals who will come in contact with the parasite later in life will have a reaction of naturally occurring cross-reactive immune cells, which will induce a strong inflammatory response. This will increase the risk of cerebral malaria and other severe manifestations. After several repeated infections a malaria specific immune response will have been developed and the parasites can now be controlled with a non-harmful immune reaction. Children will probably have fewer cross-reactive T cells, and will therefore have a lower risk of cerebral or severe malaria than adults. If the individual leaves the endemic area for some years and then returns, the immunity is lost and the individual will encounter malaria as a non-immune individual again [88].

The importance of antibodies in protection against malaria

Innate immune mechanisms involving mononuclear phagocytes and NK cells play an important role early in malaria infections. However, the importance of antibodies in protective immunity against *P. falciparum* infection was demonstrated by the classical experiments of Cohen and McGregor [108], in which passive transfer of IgG from adults had curative effects in children. In Thai individuals, passive transfer of cytophilic antibodies was associated with protection [109]. As depicted in figure 11, there are several potential mechanisms by which antibodies can neutralize the parasite (as reviewed by Bolad and Berzins [110]). Antibodies may inhibit merozoite invasion, either by neutralization of the free merozoites or by interference with the merozoite invasion process. Antibodies may also react with parasite-derived antigens expressed on the surface of iRBC, thereby inhibiting the intraerythrocytic development of the parasite. The leaky membrane of infected erythrocytes just prior to merozoite release, may give the antibodies access to the intraerythrocytic parasite, and they may interfere with merozoite dispersal. Another pathway for antibody attack may be the

parasitophorus duct, which has been suggested to form a connection for direct access of serum macromolecules to the parasite [110]. Even though antibodies can inhibit parasite invasion/growth on their own, a major effect of malaria specific antibodies is thought to be induction of antibody dependent cell-mediated inhibition (ADCI) [111] and the secretion of monocyte-derived mediators [112]. The main players in this type of killing are the FcR on the surface of the effector cells, which will bind the Fc part of the antibodies, while the Fab part of the antibody is bound to antigens on the surface of merozoites [109] or late stage infected RBC [113]. Several studies have shown that high circulating concentrations of malaria specific IgG are related to protection from severe malaria, and seroepidemiological studies in different endemic areas have demonstrated the association of IgG antibodies of the cytophilic subclasses IgG3 and IgG1 with protection against *P. falciparum* malaria [114, 115]. This association is, however, quite inconsistent when considering antibody responses to single malaria antigens. IgG3 is the major subclass in responses against *P. falciparum* antigens showing a high degree of diversity, e.g. merozoite surface protein (MSP)-2 [109], while responses against more conserved antigens, e.g. the C-terminal part of MSP-1, are dominated by IgG1 [116]. Interestingly in some populations, IgG2 is related to protection, so it is not clear what IgG subclass profile that shows highest association with protection.

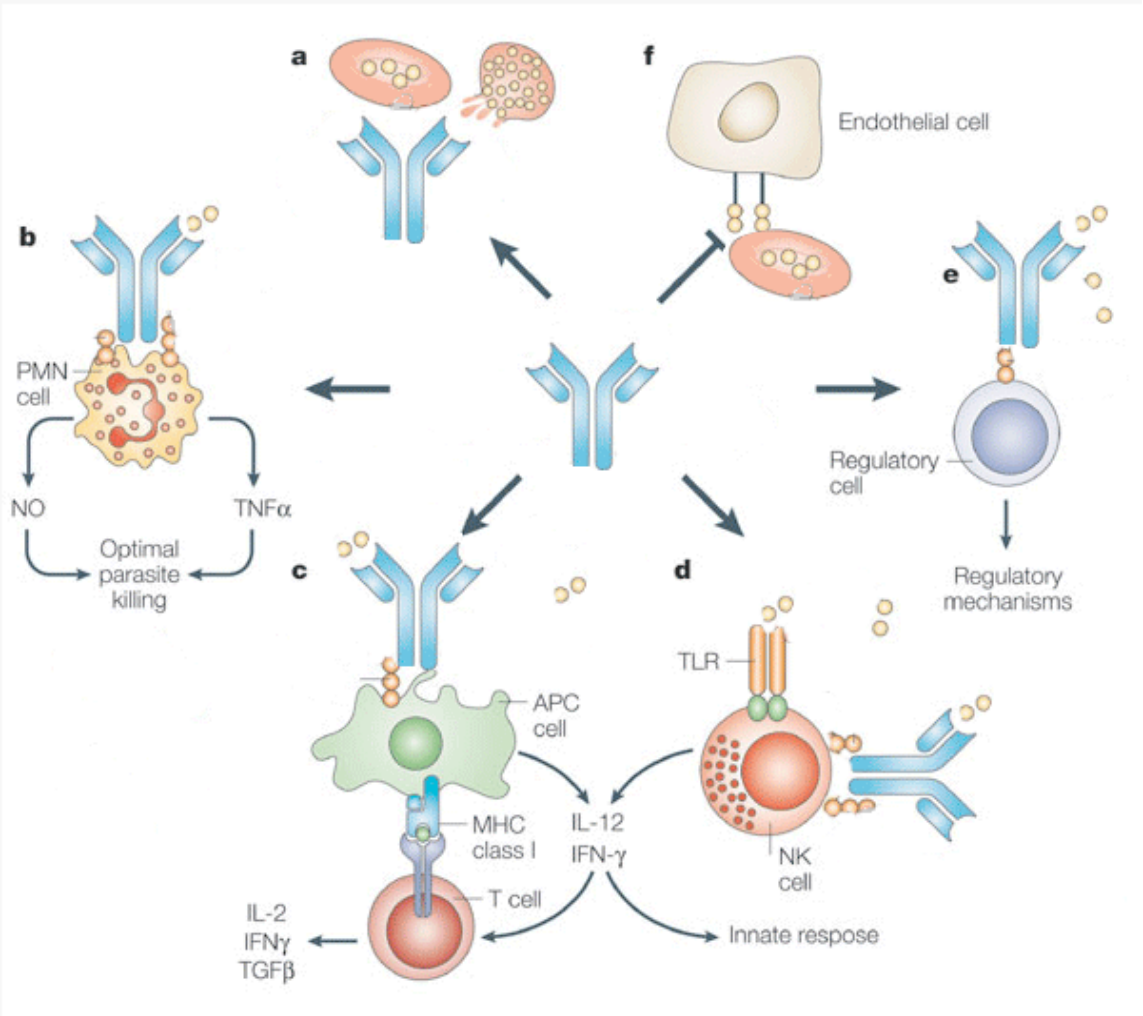


Figure 11. Antibodies reactive to malarial antigen can control the parasite in different ways. (a) Malaria reactive antibodies can bind to antigens on the merozoite or the infected erythrocyte surface; this can inhibit the entry of the merozoite into the erythrocyte. (b) Antibodies can bind to the respective FcR on a variety of cells and optimize the cells for parasite killing. (c, d). Binding of antibodies to DC and NK cells can induce strong innate responses. (e) Regulatory cells can also be affected by malaria-reactive antibodies, thereby affecting the immune reaction. (f) Antibodies can also prevent pathology by blocking the interaction of EMP1 with endothelial cell receptors, such as CD36 or ICAM-1 Adapted from Pleass *et al* 2005 [117].

Important antigens

The most important antigens being targeted by parasite neutralising antibodies are mainly expressed during the merozoite stage or the later trophozoite stage (fig. 12). At the merozoite stage, the MSP antigens, antigens present in the apical complex organelles of the merozoites (EBA-175, Rhop 1-3, RAP 1-3 and AMA-1) and Pf155/RESA, have all been shown to be targets to antibodies with the capacity to inhibit merozoite invasion (reviewed by Berzins and Anders [118]). Also, the recently identified SURFINs [119] are found in this stage. There are several antigens synthesised during the trophozoite development (e.g. GLURP, SERA, ABRA, PfEMP1, Pf332, RIFINs, STEVORs), and antibodies to several of these antigens show a high capacity to inhibit parasite growth or invasion [120-123]. Immunity to malaria is

parasite and strain specific, and clonal antigenic variation is common in *P. falciparum* [124]. The mechanism behind this antigenic variation is still not clear, but one very likely hypothesis is that there is a frequent ongoing switching of variant surface antigens (VSA) in the parasite population, and an outgrowth of one of these subpopulations would occur when antibodies are being raised towards the other VSA presented. The importance of VSAs in immunity can be shown by correlating the range of different anti-VSA antibodies to protection [125]. PAM is caused by accumulation of iRBC in the placenta. These parasites express a PfEMP1 VSA that binds to chondroitin sulphate A (CSA), and the immune responses that are confined to pregnant women are parity dependent [126]. Some important antigenically variable antigens are PfEMP1, RIFINs, STEVORs and SURFINs.

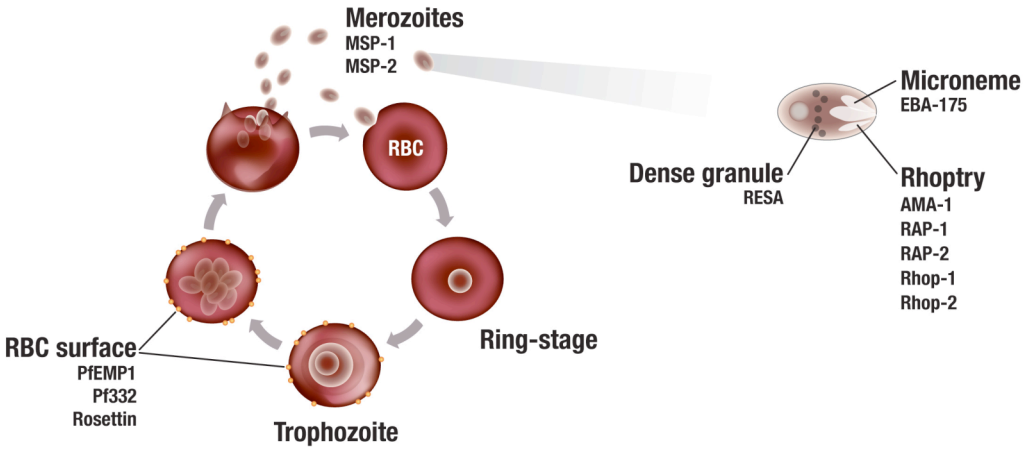


Figure 12. The location of some important antigens involved in parasite neutralizing immune responses during the erythrocytic cycle.

Host genetics and protection against malaria

Throughout the genome many alterations are present, mutations and polymorphisms being permanent heritable changes in the genetic code of all nucleated cells in the individual. These are called polymorphisms if present at a frequency of 1 % or more in the general population, or germline mutations if present in less than 1 % of the population. Single nucleotide polymorphisms (SNPs) are usually silent changes or found in intronic sequences, and it has been estimated that our genome contains about 2 million SNPs. Somatic mutations are non-heritable acquired alterations only present in the affected tissue.

Several polymorphisms have shown both harmful and protective qualities, e.g. sickle cell anaemia, a defect in the RBC that confers protection against severe malaria, while also making them less effective in transporting oxygen.

With the environmental factors still being believed to contribute the major risk in malaria, why then study genetic variations? A study performed by Mackinnon *et al* (2005) [128] tried to answer this very valid question. This study showed that genetic and undefined household factors are responsible for approximately one quarter each of the variability in malaria incidents, and the known protection from HbAS cannot, by it self, explain this huge influence of the genetics of the host. The long history of co-evolution of humans and *Plasmodium* parasites do give room for the speculation that the parasite would have had a major influence on the selection for more protective haplotypes in humans living under a strong malaria pressure, giving them a more favourable immune response fighting this parasite. However, this specialization seems to critically increase the risk of MS and other autoimmune diseases [129]. A connection between malaria and MS has been shown [130]; suggesting that what is a favourable genetic pattern in malaria is non-favourable in MS. This can be seen in the population of Sardinia where malaria was abruptly eradicated and different autoimmune disorders are very prevalent today [131].

Red blood cell variations

Studies of genetic influences on the susceptibility to a malaria infection, have, not surprisingly, shown that the majority of the malaria resistance genes are related to the structure or function of the RBCs. The most common protective genetic variants are in the genes related to sickle cell trait, thalassemias, enzyme deficiencies, ovalocytosis and the ABO blood groups. Although these variations account for a large part of the protection against malaria from genetic factors, they are not enough to explain the variations in malaria severity seen between individuals living in malaria endemic locations.

Ethnic differences in genetic background

Several studies have demonstrated differences in susceptibility to malaria between different ethnic groups. The Tharu people in Nepal and the Orang Asli in Malaysia both showed a lower susceptibility to malaria as compared to other ethnicities in the same areas [132, 133], and in West-Africa, the Fulani showed a higher frequency of splenomegaly and lower incidences of malaria than other sympatric groups, despite the same exposure to malaria and no apparent differences in socio-cultural circumstances [134]. This finding was later confirmed by various studies, showing that the Fulani have a lower parasite prevalence and density and have a more prominent spleen enlargement compared to other ethnic groups [135, 136]. Moreover, the Fulani have generally higher anti-malarial antibody responses, including antibodies against both liver stage [137, 138] and blood stage antigens [138, 139], as well as

against crude *P. falciparum* blood stage extract [136]. Human leukocyte antigen (HLA) analyses have shown that the Fulani are genetically distinct from other African ethnic groups [140]. Furthermore, established genetic malaria resistance factors, like haemoglobin S and C, alpha thalassemia, G6PD and HLA B, have been shown to occur at a lower frequency in the Fulani than in their sympatric neighbours [136, 141]. The proportion of individuals not having any of these protective alleles was more than 3-fold greater in the Fulani, as compared to the other ethnic groups [141]. IL-4 levels and polymorphisms have been suggested as one contributing factor to this lower susceptibility to malaria in the Fulani [101, 142], but these findings are not enough to explain these ethnic differences. Thus, many studies are ongoing, covering a wide range of possible effector functions, e.g. cytokine expression, TLR expression, cell activation and receptor functions.

Polymorphisms in Fcγ receptor genes

The Fcγ receptors on monocytes and other leukocytes are important structures in the immune defence against pathogens, since the binding of antibodies to the Fc-receptors provokes biological functions as e.g. antibody dependent cell cytotoxicity (ADCC), ADCI and phagocytosis [143]. Polymorphisms that change their function or distribution have been reported (fig. 13) [144], and this could have an influence on the susceptibility to different infections, such as malaria, where it has been known for many years that IgG antibodies are important in the protection and clearance of the infection [108, 114]. Moreover, immune-complexes acting through FcγRII induce IL-6 and IL-10 production from peripheral blood mononuclear cells (PBMC) [145], two cytokines which could affect the outcome of the malaria infection.

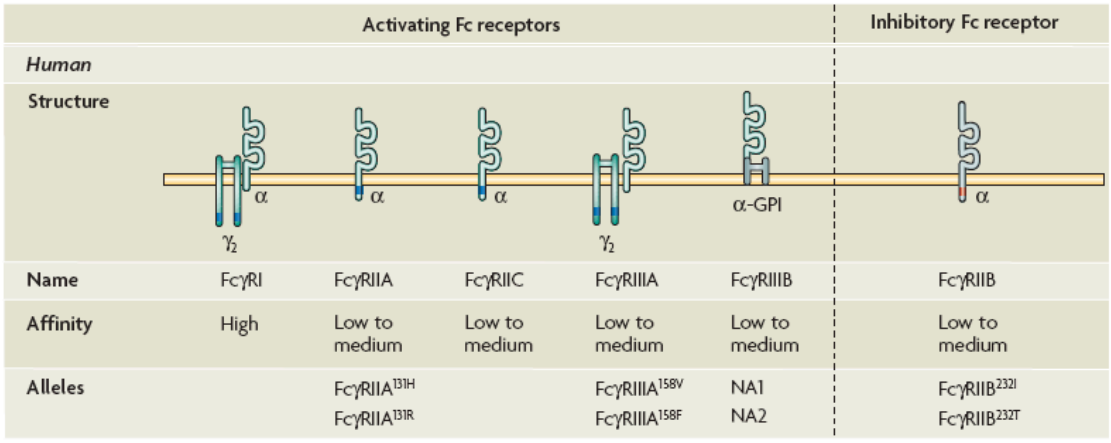


Figure 13. The family of human Fcγ receptors with the polymorphisms that has been analysed in this thesis, except for FcγRIIc Q13 STP. Adapted from Nimmerjahn 2008 [57].

The FcγRIIa has a dimorphism in amino acid 131, resulting in an arginine (R) to histidine (H)

substitution. The H-allotype receptor is the only efficient receptor for IgG2 [146]], and that same allele has in some previous studies been associated to an increased susceptibility to malaria (reviewed by Braga *et al* [147]). However, there are also a number of studies reporting the opposite, *i.e*. an association of the H-allotype with protection from malaria [148-152]. As, mentioned previously, CRP may bind to FcγRIIa, and, importantly, the R131 receptor allotype shows higher affinity in this regard [153].

The FcγRIIb is the only inhibitory FcγR, capable of inhibiting various aspects of the immune response, e.g. antibody responses, cytokine production, antigen presentation and phagocytosis [154]. FcγRIIb-deficient mice have been shown to have a reduced *P. chabaudi chabaudi* parasitemia and disease severity [155]. A substitution within the transmembrane domain encodes a threonin (T) for isoleucine (I) substitution at position 232, and FcγRIIb 232T is found at a higher frequency in areas where malaria is endemic, suggesting a protective influence of this allele on malaria [155].

FcγRIIc is expressed on NK cells, and a change from glutamine (Q) to a stop codon at amino acid position 13 in exon 3 affects the expression of the receptor [156], which could have an effect on the NK cells [157].

FcγRIIIa has a dimorphism at amino acid 176, changing a phenylalanin (F) to a valine (V) in the membrane proximal extracellular domain, encoded in exon 4. The V/V allotype receptor has shown a higher affinity for IgG1, IgG3 and IgG4 than the F/F allotype receptor [158]. In malaria, so far, no associations have been shown between this polymorphism and severity of the malaria infection [159].

FcγRIIIb can occur as neutrophil antigen (NA) 1 or 2, differing in 4 amino acid positions. NA2 homozygozity is related to a lower phagocytic capacity. NA2, in combination with FcγRIIa 131 H/H, has been associated with an increased susceptibility to cerebral malaria [160].

C-reactive protein gene polymorphisms

As mentioned previously, CRP is an acute phase protein that increases rapidly in the circulation during infections and inflammations. Studies on *P. falciparum* malaria have

related high circulating CRP levels with parasite density and severity of the malaria infection [161, 162], while in individuals with asymptomatic *P. falciparum* infections, plasma levels of CRP are low [163, 164]. The role of CRP in *P. falciparum* infection is not clear, CRP has been associated with complement mediated haemolysis of infected erythrocytes and subsequent anaemia [165], but was also implicated in the defence against pre-erythrocytic stages of malaria [166]. Importantly, CRP induces the anti-inflammatory IL-10 [26], which could affect the early immune responses seen in a malaria infection.

Several polymorphisms have been reported in the CRP gene (fig. 14), however only three of them are showing convincing functional effects (-717, -286, +1444), although the most promising functional polymorphism would be the -286 (rs3091244). This polymorphism in the promoter region of the *CRP* gene is tri-allelic (C>T>A) and it has been strongly associated with the circulating concentrations of CRP [167].

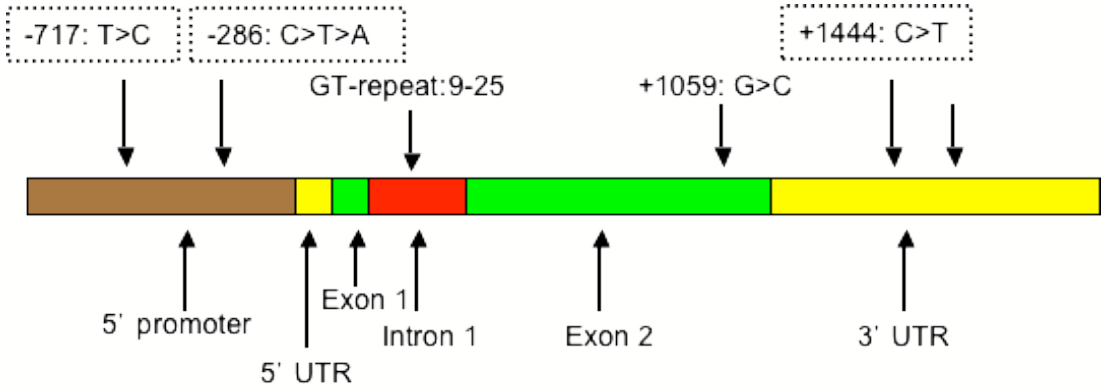


Figure 14. Some of the most studied polymorphisms in the *CRP* gene, with the three analysed in this thesis highlighted.

Cytokine gene polymorphisms

Cytokines are important players in the immune responses and polymorphisms in cytokine genes can have effects on many down stream functions. The levels of CRP are easily affected by cytokines; hence SNPs in different cytokine genes could affect the circulating levels of CRP. Also, important functions related to clearance/control of different pathogens could be severely affected.

High levels of IL-6 increase the risk of a fatal outcome in a malaria infection [168], but IL-6 and its soluble receptor sIL-6R can regulate proliferation and damage in hepatocytes [169], and IL6 also functions as a B-cell stimulatory factor to induce antibody production [61]. IL-6 is also a very potent inducer of CRP [61], and two polymorphisms in the IL-6 promoter region, -174 G/C and -634 G/C, strongly influence the circulating CRP levels [170].

As mentioned earlier, IL-10 may limit the production of proinflammatory cytokines and chemokines, and can thereby regulate the inflammatory response during an infection [171], thus promoting a lower clearance but with limited symptoms [64, 172]. The up-regulation of Treg in a malaria infection has been shown to increase the parasite growth [173], and the involvement of IL-10 in the generation of peripheral Treg [66] is interesting findings. A recent study demonstrated that the Fulani present a functionally impaired Treg repertoire as compared to their sympatric neighbours [174]. Three functional polymorphisms in the promoter region of the IL-10 gene (-1087, -819, -592) have been associated with the expressed levels of this cytokine [67, 68] and these could have an influence on the malaria infection progress.

IL-1β is a strong activator of the acute phase response and inflammation and it has been related to the pathogenesis of many diseases. Three gene polymorphisms in the promoter region (-3737, -1464, -511) predict the clinical levels of IL-1β and CRP [60], and a recent study from Kenya associated the haplotype -31C/-551A with an increased risk of severe malarial anaemia and lower circulating IL-1β levels [175].

TNF is also a stimulator of the acute phase response, and it has also been related to the pathogenesis of complicated *P. falciparum* malaria. TNF has been shown to be elevated in patients with *P. falciparum* malaria, and high levels of TNF correlates with disease severity and death [176]. The gene for TNF is highly inducible and, polymorphisms in the promoter region could therefore have an effect on TNF production. Several polymorphisms have been studied with regard to this hypothesis and four of them (-1031, -863, -308, -238) have been defined as strong influencer of TNF levels and different disease outcomes [177-181].

Effects of co-infection with helminths, viruses and bacteria

Helminths are very prevalent in areas where malaria is common, and co-infections are very common and deserve attention, since they could have a great impact on each other. Several studies suggest that a helminth infection can increase the susceptibility to *P. falciparum* infections. In children with a high exposure to both *P. falciparum* and *Schistosoma mansoni* there was a considerable exacerbation of splenomegaly, and despite anti-schistosome treatment the increase in spleen enlargement remained high [182]. In contrast, a protective

effect of infection with *S. haematobium* on *P. falciparum* malaria has been reported, so the influence on susceptibility to malaria seems to be highly influenced by the type of helminth, age of the individual and intensity of the infection [182]. The immune response induced by the acute phase of an *Schistosoma* infection is pro-inflammatory, while after egg deposition it is shifted towards an anti-inflammatory response. This could have severe effects on the outcome of a *P. falciparum* infection, since a to high pro-inflammatory response will mediate more severe malaria pathology, and a too fast control of the pro-inflammatory environment will result in a uncontrolled parasitemia [182]. Interesting reports of a protective effect of *Ascaris lumbricoides* have shown that, in individuals with a co-infection of *P. falciparum* and *A. lumbricoides,* there are less circulating *P. falciparum* parasites. This could have a impact on the development of cerebral malaria due to the assumable lower number of parasites ready to sequester [182].

Co-infections of malaria parasites with different viruses and bacteria have not been as well studied. One of the most severe human diseases, HIV/AIDS, has an overlapping geographical distribution with malaria, and studies have been performed to elucidate how these two infections could affect each other. So far, no conclusive results have been presented (reviewed by Rénia and Potter 2006 [183]), but since HIV infects CD4+ T cells, important for development and maintenance of anti-malarial immunity, it would be expected that these two infections could interfere with each other. Another severe infectious disease with similar geographical distribution is tuberculosis, and it has been showed that co-infected mice where less likely to contain the *Mycobacterium tuberculosis* infection and had increased mortality [184]. However, the understanding of the interactions between malaria parasites and *M. tuberculosis* and other bacteria is very limited [185].

The present study

Aim of the thesis

It has been known for a long time that antibodies confer protection against malaria. However, the isotypes of the antibodies and by which mechanism they function is still unclear. Moreover, other factors could inhibit the effect seen by antibodies, and if these are not identified, the results will be misleading. In this thesis the aim was to identify new aspects of antibodies as protective agents in malaria infections. With a genetic approach we tried to describe important pathways related to the action of antibodies in the complex immune response during an infection. In short, paper I and II are investigating antibody patterns, paper II-V genetic variations that could have an effect on malaria susceptibility.

The specific objectives were:

- To define the patterns of antibodies that could have a protective effect on malaria infections, paper I and II aimed to investigate the possible ethnic differences in malaria and non-malarial pathogen reactive antibody concentrations as well as total (paper I) and to define the IgG subclass pattern in both malaria reactive and total IgG subclass antibodies (paper II).
- The Fcγ receptors mediate many of the biological effects of antibodies, and these actions could be affected by polymorphisms in the genes coding for the receptors. Paper II and III aimed at investigating possible ethnic differences in the FcγRIIa R131H polymorphism (paper II) and the FcγRIIb T232I, IIc Q13Stop, IIIa F176V and IIIb NA1/NA2 polymorphisms (paper III) and their possible roles in IgG subclass distribution and malaria susceptibility.
- CRP is an acute phase protein that can bind to the FcγR and might thereby have a blocking effect on the binding of IgG antibodies. The levels of CRP are difficult to measure, but functional polymorphisms in the CRP gene as well as several cytokine genes have shown an influence on the concentration of CRP. Paper IV and V aimed at investigate the influence of the CRP -286 C/T/A, -717 T/C and +1444 C/T polymorphisms (paper IV) on malaria susceptibility, and investigate the influence of

polymorphisms in cytokine genes (Il-1β, IL-6, IL-10 and TNF) on CRP and susceptibility to malaria (paper V).

Mali

Country profile and health status

Mali is a vast, semi-arid, landlocked country covering 1.2 million km^2, of which 60% is desert. It is a vast land of plains fed by two major rivers: the Senegal on its western edge and the great River Niger. Mali's economic growth was 5.3% per year on average for the 2003-06 period, driven primarily by gold mining, and transport and telecommunication services. Despite favourable economic growth, Mali remains one of the world's poorest countries, rated 173 out of 177 countries (UNDP Human Development Index 2007/2008), and poverty remains mainly a rural problem. Mali's population of 12 million is predominantly rural, with a growth rate of 2.7% a year. Life expectancy is 53 years, 48% of the population are younger than 15 years, 49% are between 16 to 64 years and only 3% are older than 65 years. The maternal mortality ratio is 1200 deaths per 100 000 live births, the infant mortality rate is 121 deaths per 1000 live births, and the under 5 year mortality is 219 per 1000 live births (WHO Country Health System Fact Sheet 2006, www.who.int/countries/mli/en/). The major causes of death in children less than 5 years of age are neonatal causes, pneumonia, malaria and diarrhoeas, which are also the most common causes for all ages (WHO Country Health System Fact Sheet 2006, www.who.int/countries/mli/en/).

The presence of malaria differs in the country, it is absent in the northern parts and endemic in the southern parts, and the transmission season varies from north to south (fig. 15).

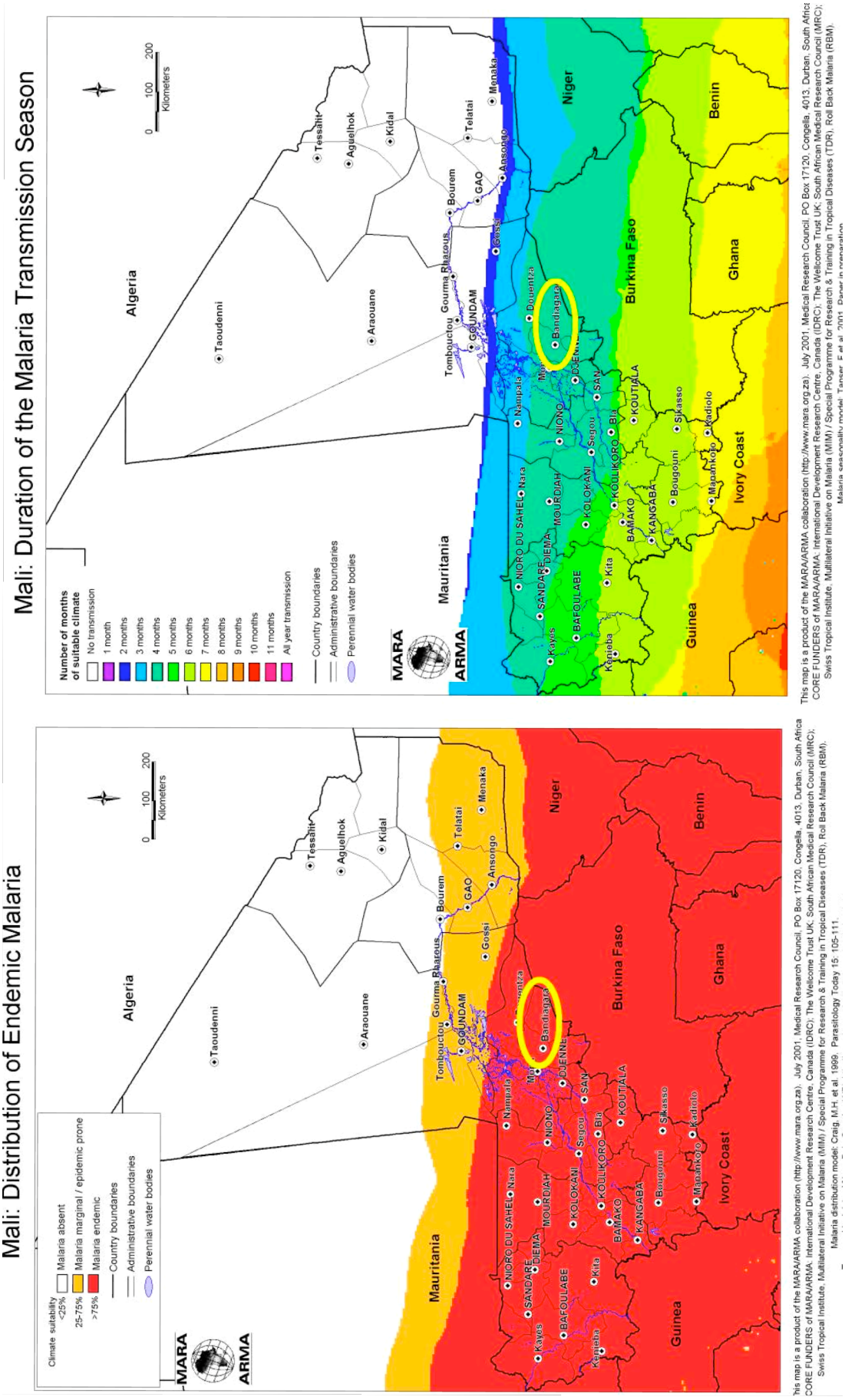


Figure 15. Distribution of endemic malaria and the duration of the transmission season in Mali. The area where the sample collection took place is marked with a yellow circle. Adapted from MARA/ARMA (www.mara.org.za).

The malaria situation in the study area

The participating villages in Mali were Mantéourou, Naye, Binédama and Anakédié, located approximately 850 km from Bamako close to Bandiagara (fig. 16). The Mantéourou and Naye are divided into two subdivisions, Mantéourou Peulh and Mantéourou Dogon and Naye Peulh and Naye Dogon-Dinsogou. The two subdivisions are separated by 300-500 meters and inhabited by either the Fulani or the Dogon ethnic groups. The other two villages, Binédama and Anakédié, are exclusively populated by either Fulani or Dogon, respectively, and are located approximately 1000 meters apart. The transmission in this area is meosendemic with a rainy season extending from June to October. The mosquito infection rate (MIR) and the entomologic inoculation rate (EIR) are rather similar between the villages [136]. The main vector in this area is *An. gambiae* s.l with a minor contribution of *An. funestus* [136]. The use of bed nets has been reported to be at the same frequency in the two ethnic groups living in the area, and episodes of clinical malaria were more frequent in the Dogon ethnic group than in the Fulani [136]. Intestinal parasitosis was more frequent in the Fulani, while other diseases, such as pulmonary infections, diarrhoeas, rheumatologic, dental, ophthalmologic, and dermatologic disease, were found at the same incidence frequency in the two ethnic groups [136].

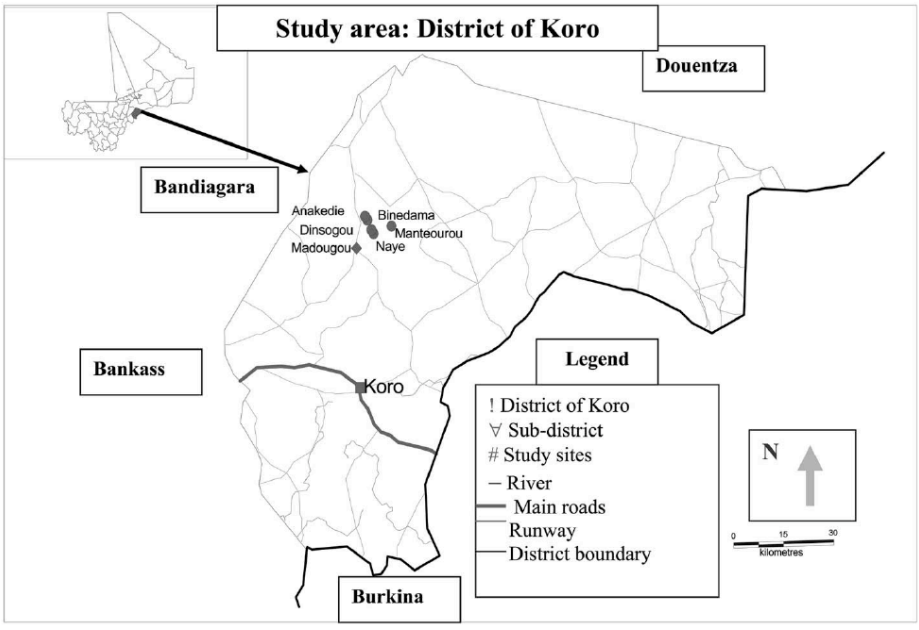


Figure 16. The study area in Mali. Adapted from Dolo et al. 2005 [136], reprinted with permission from the American Journal of Tropical Medicine and Hygiene

No intermarriages occur between the Fulani and the Dogon in these settings, and although these villages are sharing the same environment, some cultural and social differences are present; Fulani are cattle breeders while Dogon are farmers. This has some effects on their

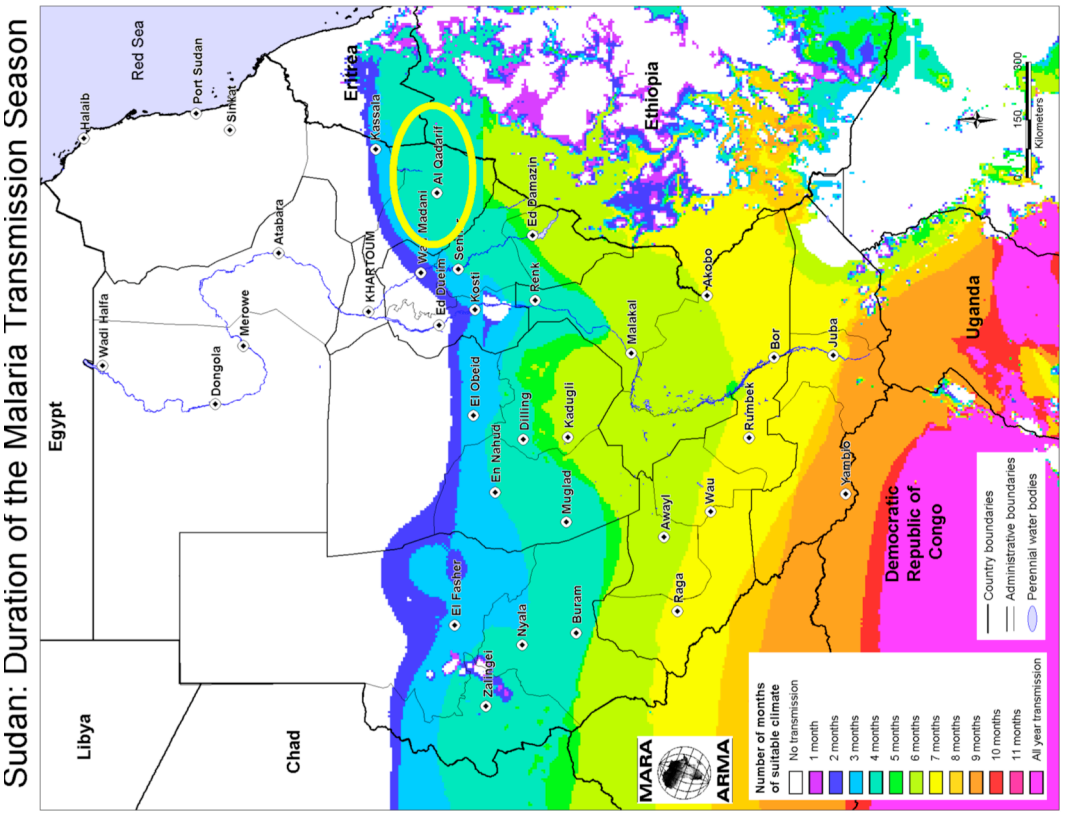
diets, with the Fulani eating more milk-based products than the Dogon (Prof. A. Dolo, personal communication).

Sudan

Country profile and health status

Sudan is situated in the north-eastern corner of Africa, and it is the largest country on the African continent. Despite its internal conflicts, Sudan has a good economic growth, and is being ranked as 147th out of 177 countries (UNDP Human Development Index 2007/2008). The majority of the Sudanese population of 40 millions is rural, with a growth rate of 2.5% a year. Life expectancy is 57.4 years, 41.7% of the population is younger than 15 years, and 4% is older than 65 years. The maternal mortality rate is 590 deaths per 10,000 live births and the infant mortality rate is 62 deaths per 1000 live births, and the under 5 year mortality rate is 91 deaths per 1000 live births (WHO Country Profiles, www.emro.who.int/sudan). The major causes of death in children under five years of age are neonatal complications, malaria, pneumonia and diarrhoeal diseases, while the top causes for death in the overall population is ischemic heart disease, malaria, HIV/AIDS and diarrhoeal disease (WHO, Mortality Country Fact Sheet 2006, www.who.int/countries/sdn/en/).

The malaria transmission differs from the north to the south. In the northern, eastern and western states, malaria is mainly low to moderate with predominantly seasonal transmission and epidemic outbreaks. In southern Sudan, malaria is moderate to high or highly intense, generally with perennial transmission (fig. 17).



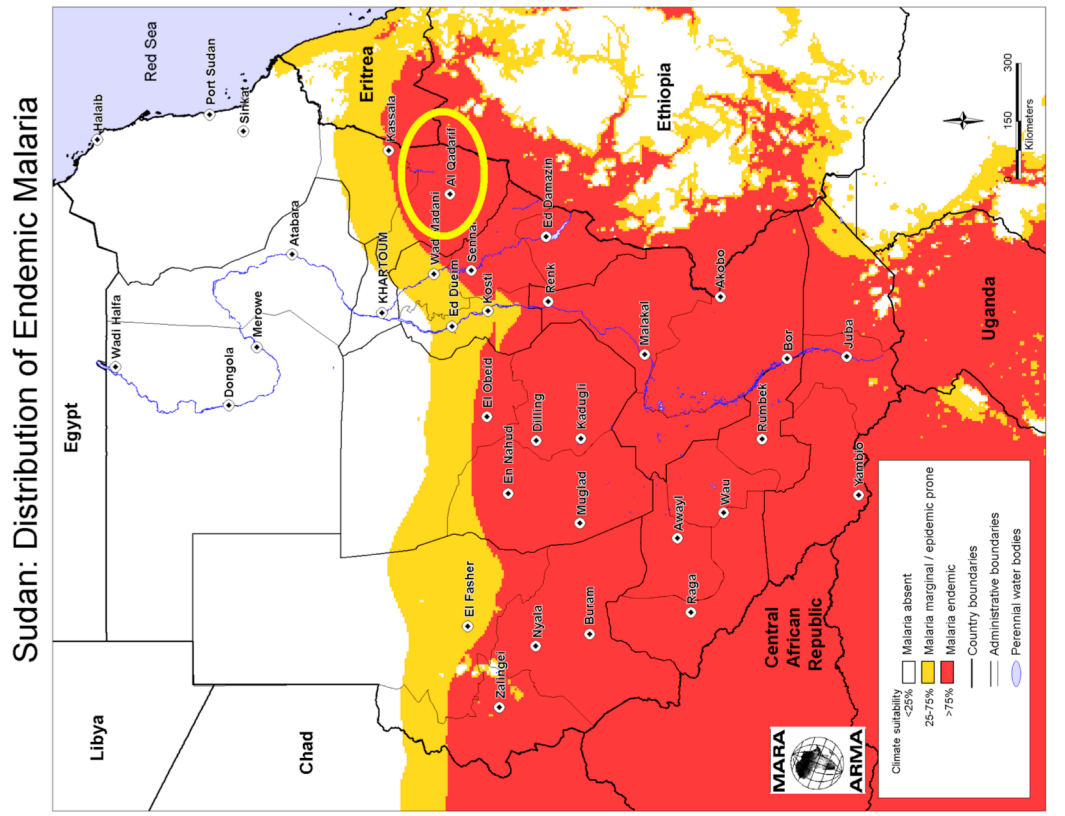


Figure 17 Distribution of endemic malaria and the duration of the transmission season in Sudan. The area where the blood collection took place is marked with a yellow circle Adapted from MARA/ARMA (www.mara.org.za).

The malaria situation in Daraweesh

The study area in Sudan is located in the Daraweesh village in the Gedaref State in eastern Sudan, 450 km from Khartoum and 16 km from the Gedaref town. Malaria transmission is markedly seasonal and unstable in this area, with more than 95% of the yearly cases occurring in September – November [186] and the annual peak parasite prevalence ranges from 1 to 40% in different years [187]. The EIR has been estimated to 2 infected bites/year, the transmission is hypoendemic and *P. falciparum* is responsible for more than 96% of the malaria cases [186].

The majority of the people in Daraweesh are descendants of migrating Fulani from Burkina Faso, reaching Daraweesh around 120 years ago [186].

Comments on methodology

Some of the methods used in the included papers deserve a more detailed description of the theory behind them. This section also comments upon the statistics used in the papers, the ethical permissions and informed consents obtained in the studies.

Pyrosequencing™

To genotype a tri-allelic polymorphism can be complicated. If using common restriction enzyme methods, it usually requires two or more enzymes and the results will be highly subjected to errors during the analysis. Instead, we used pyrosequencing™ in order to obtain a qualitative analysis of the tri-allelic polymorphism analysed in paper IV. Pyrosequencing™ is a non-electrophoretic method for DNA sequencing, based on the "sequencing by synthesis" principle. It relies on an enzymatic cascade that starts with the release of pyrophosphate (PPi) as a result of nucleotide incorporation by the Klenow fragment of DNA polymerase I. ATP sulfurylase will convert the released PPi to ATP, which will facilitate the oxidation of luciferin and generate light. Since the added nucleotide is known, the template sequence can be determined. A pyrase is included in the pyrosequencing reaction in order to degrade unincorporated nucleotides and excess ATP between the base additions. If not included, these will disturb the light signals from the incorporated nucleotides. In short, the technique is performed as follows: The template DNA is amplified using a biotinylated primer and the product is subsequently immobilised with streptavidine coated sepharose beads. A careful wash removes salts and other PCR leftovers, followed by a pH change that results in single-stranded PCR products. After hybridization of a specific sequencing primer, the obtained template is run on a pyrosequencing apparatus. The obtained result file is a pyrogram, in which the sequence of the template can by viewed.

Taqman® MGB probes

In paper IV and V, Taqman® MGB probes were used. This enabled us to run the analyses in 384-well plates, which limit the time and handling, and moreover this method is, if designed

properly, highly accurate and easily interpreted. The Taqman® MGB probes from Applied Biosystems rely on the specificity of the two probes recognizing the two different alleles. The probes are linked to reporter dyes (VIC® or FAM™) in the 5' end and a minor groove binder (MGB) at the 3' end of the probe. The MGB modification increases the melting temperature of the probe, allowing shorter probes, which produce a more robust allelic discrimination. In short, the technique is performed as follows: During the PCR the specific probes and primers anneal to their complementary sequence, the AmpliTaq Gold® DNA polymerase extends the primers and cleaves only probes that are hybridized to the target sequence. This separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter. Thereby making it easy to discriminate between allele 1 and 2, which can be illustrated in an allelic discrimination plot.

Statistics

Hardy-Weinberg equilibrium

In paper II-V the frequencies of the alleles were checked for Hardy-Weinberg equilibrium (HWE) using a χ^2-test. The Hardy–Weinberg principle is used in population genetics and states that the genotype frequencies in a population remain constant or are in equilibrium from generation to generation. A statistically significant deviation from the HWE could indicate that the population in question is subjected to non-random mating, selection pressure, random genetic drift or gene flow. Sometimes it is also used to control if the genotyping is correctly performed, since it is very rare to find alleles deviating from HWE.

Non-parametric tests

When the obtained data is not normally distributed, all analyses should be done with non-parametric tests. These tests are more robust for differences in the distributions within the data and will therefore not be affected by this. Mann-Whitney U-test is used when comparing two groups, and does not require normally distributed data, however it does assume that the two distributions are similar. It is almost impossible to identify a similar distribution in a sample population, therefore it is mostly a theoretical issue, but if it is known before that the two populations are different in their distribution, another test should be used. In paper I we used non-parametric tests to detect differences in levels of malaria reactive as well as total antibody levels.

Parametric tests

When the data is normally distributed, a parametric test can be used. Parametric tests require that the data is normally distributed and that the variances are similar. These requirements are rarely fulfilled completely, but most parametric tests can hold for small deviations between the groups. It is commonly accepted to normalize data that is not normally distributed and the easiest is by log-transformation. This could make the data normally distributed and also stabilize the variances. In this thesis, the data in paper II was transformed, which made it possible to use parametric tests for the analyses. This simplified the analyses and made the result more robust since fewer tests could be used and thereby limiting the probability of presenting false-positives.

Correction for multiple tests

When testing the same variables in multiple independent tests, it is advised to correct for the number of tests you perform, otherwise it is possible to present false-positives. The term statistically significant actually means that a given result is unlikely to have occurred by chance. The Bonferroni correction is the easiest and mostly used correction method, and it is simply done by dividing the chosen significance level with the number of tests performed on the same data. For example, to test two independent hypotheses on the same data at a 0.05 significance level, one would use the stricter threshold of 0.025 if corrected by Bonferroni.

Regressions

Regression analysis is used when considered necessary to predict a dependent variable from a number of independent ones. If more than one independent variable is known, multiple regressions can be used. When using regressions, it is important to remember that we can say that variable X predicts Y, we cannot say variable X causes Y.

Association analysis using UNPHASED

UNPHASED is a software for performing genetic association analysis in both families and unrelated individuals. It implements maximum-likelihood inference on haplotype and genotype effects while allowing for missing data. UNPHASED is distributed free of charge under the GNU public license, and it is developed by Frank Dudbridge [188],

http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/. Paper II-V used UNPHASED (version 2.403) to analyse associations of genotypes and haplotypes.

Ethical permissions

Ethical clearance of the studies were obtained from: The Ethical committee of the Faculty of Medicine and Pharmacy of Mali, the Ethical committee of Karolinska Institute, Sweden, The Ministry of Health of Burkina Faso, and the Ethical Committee of University of Khartoum and national clearance from the Sudanese Ministry of Health.

Informed consents were obtained from all individuals or their guardians included in the studies. In Mali this was done in a two-step manner. First an oral community consents, in which the investigators explains the aim of the study to the inhabitants of the village, the chief of the village and the healers. Second, the individual consents are obtained for each participating individual just prior to the blood collection, an alphabetized volunteer puts his signature and the non-alphabetized signs with his or hers finger print. This procedure was similar in Burkina Faso and Sudan.

Results and complementary discussions

Antibody levels and patterns of malaria reactive antibodies in relation to malaria susceptibility

The well-known relative resistance to malaria seen in the Fulani ethnic group, as compared to other sympatric ethnicities, has been related to the higher concentrations of anti-malarial antibodies seen in the Fulani [139]. The ability to mount a stronger anti-malarial immune response was suggested to be at least in part genetically regulated [101], and this is the main focus of this thesis. However, in order to evaluate antibody responses in this context it is important to assess if it is a malaria specific phenomenon we are studying or a general activation of the immune system.

Therefore, we first investigated if Fulani individuals are generally more reactive also to antigens from other pathogens than malaria parasites, or if it is a pathogen-specific reaction. We designed a panel of non-malarial antigens, including the measles and rubella viruses, *Toxoplasma gondii*, *Helicobacter pylori*, and a *Mycobacterium* antigen (PstS-1 [189]). The IgG antibody responses against these antigens were analysed, as well as the total concentrations of IgM and IgG in Fulani and non-Fulani individuals from Burkina Faso and Mali (I). Furthermore, the total IgG subclass concentrations were determined in individuals from Mali (II). Higher concentrations of IgG reactive with the measles and *T. gondii* antigens in the Fulani compared to the other ethnic groups were detected in both countries. Only the Malian Fulani showed higher antibody levels against the *Mycobacterium* PstS-1 compared to their sympatric tribe, while no difference was seen in Burkina Faso for this antigen. For rubella and *H. pylori*, no differences between the ethnic groups were seen. All antigens, except the recombinant PstS-1, were crude heat inactivated extractions, making it possible to assume that native antigens were detected. Measles and *T. gondii* are common infections in these settings and the results obtained for these two antigens suggest that these two share epitopes with *P. falciparum*, and that cross-reactive antibodies might have been detected in our analyses. These findings suggest that the Fulani are not hyper-responsive to all antigens, but also that the higher IgG responses are not exclusively specific for malaria. Measles and *T. gondii* may also show cross-reactivity with the soluble schizont antigens from *P. falciparum*, which also has been shown to signal through TLR9 [190-193]. Thus, polymorphisms in TLR9

[194] can be a contributing factor for the differences in anti-malaria response seen between Fulani and their sympatric neighbours.

While the total IgG concentrations did not differ between the two ethnic groups in either country, total IgM levels were shown to be higher in Fulani than in the non-Fulani (I). No differences between the two ethnic groups were shown when comparing total IgG subclasses, except for IgG4, were the Dogon showed slightly higher concentrations than the Fulani (II). This confirms in part the previous suggestion [174], that the relative resistance seen in the Fulani as compared to other sympatric ethnic groups appears to be pathogen related, but also that this higher response is not a result of a generally more activated immune system in the Fulani.

Despite a difference in transmission intensity, Fulani from Mali showed similar levels of *P. falciparum* specific IgG, IgM and IgG subclasses as the Fulani from Burkina Faso (I). Fulani from both Burkina Faso and Mali had higher levels of all malaria-specific antibodies when compared with those of the respective sympatric ethnic groups. The higher levels of anti-malarial IgG and IgM in the Fulani groups, suggest a role of these antibodies in the lower susceptibility to malaria seen in Fulani. This is in line with previous studies, suggesting a role of malaria specific IgG and IgM in the defence against malaria [108, 195]. It has been shown that B cells expressing IgM can persist long after the malaria transmission seasons [195, 196], which could be further supported in our results by the higher total concentrations of total IgM in Fulani as compared to non-Fulani groups.

Anti-malarial IgG subclass antibodies have been shown to be important in malaria immunity, in particular, antibodies of the cytophilic IgG1 and IgG3 subclasses having been related to protection [197]. The suggested mechanism by which these subclasses are protective, involves their binding to the Fc receptors on monocytes, leading to ADCI of parasite replication [197, 198]. This is supported by our results, since the two most predominant IgG subclasses in both study I and II are IgG1 followed by IgG3. However, in study II we also detected a high IgG2 response in some Fulani individuals. The ratios between IgG1:IgG2, IgG1:IgG3 and IgG2:IgG3, showed generally high IgG1, IgG2 and IgG3 responses in the Fulani groups, while the Dogon had predominant IgG1 and IgG3 responses. The previous suggestion, that IgG1 and IgG3 are the most important IgG subclasses in the protection against malaria, are mainly based on the finding that IgG1 and IgG3 can induce opsonisation of infected red blood cells, while IgG2 and IgG4 have been shown to inhibit this opsonisation.

[198]. However, some reports suggest a protective role of anti-malarial IgG2, with high IgG2 and low IgG4 levels of certain anti-malarial antibodies being associated with resistance to *P. falciparum* malaria [148]. The data reported here showed that the Fulani have higher anti-malarial IgG2 levels compared to the Dogon, while IgG4 were similarly low in the two ethnic groups. This suggests that the association of high IgG2 and low IgG4 levels reported by Aucan *et al* [148] could be a contributing factor for the relative resistance to malaria seen in the Fulani. The results in study I and II support the hypothesis by Eisenhut [199] that it is a IgG subclass pattern that is important for protection rather than the single IgG subclasses by themselves.

Overall, the differences in levels and patterns of malaria reactive antibodies could be a major reason for the lower susceptibility seen in the Fulani group. There are some possible causes for these differences, one being differences "downstream", for example the known polymorphic FcγRIIa, where the mutated protein is the only FcγR that can bind IgG2 efficiently. This could have an impact on the cytokine production by the effector cells, and thereby affect the cytokine milieu, which has a major influence on the Ig class switching. Second, "upstream" mechanisms, such as cytokine production and polarisation, have been shown to affect the Ig isotypes. Polymorphisms in cytokine genes, or other genes with an influence on cytokine production, can affect the production of the proteins and this could affect the Ig class switch.

Genetic variations and ethnicity

FcγR polymorphisms: are they relevant for malaria susceptibility?

The Fcγ receptors are important structures in the antibody related immune defence against pathogens, and functional polymorphisms in the genes coding for these receptors could influence the capacity to control or clear infections in the host. We analysed known functional polymorphisms in the genes coding for the five low to medium affinity FcγR, FcγRIIa-c, and FcγRIIIa-b, in asymptomatic individuals from Mali.

The FcγRIIa R131H genotype has shown a possible potential to influence the IgG subclass concentrations [200], and the RR genotype has been associated with protection from severe malaria in several studies [147]. However, our FcγRIIa R131H genotype results (II) are contradictory to this, since we could show that the RR genotype and R-allele is associated

with the Dogon ethnic group. The previously suggested susceptibility allele was more common in the Fulani than in the Dogon, despite a lower susceptibility to malaria in the Fulani group. This is supported by an independent study in Sudan [201], were the same results were seen; the HH genotype is more common in the Fulani. Recent reports from Brazil [152], India [151] and Sudan [149] have shown a correlation between the H allele and protection from severe malaria, further strengthening our finding. Moreover, this suggests that more factors than malaria could be influencing the 131 R/H polymorphism, and the protection associated to this. Pathogen pressure is an obvious factor that has shown a tremendous effect on genetic selection for many pathogens. Different settings could have differences in the selection for favourable alleles, depending on what other pathogens that are present and at what intensity. The FcγRIIa 131H allele will give a better protection to encapsulated bacterial infections promoting an IgG2 response [202], and even if this selection pressure seems to be low worldwide, it could be detrimental in smaller isolated populations, giving an explanation to the inconclusive results that have been published regarding the FcγRIIa R131H polymorphism.

For the FcγRIIB T232I, FcγRIIc Q13STP, FcγRIIIA F176V and FcγRIIIb NA1/NA2, no ethnic differences in genotype or allele frequencies could be detected in our material (III), giving an additional hint of the possible importance of the FcγRIIa R131H polymorphism. FcγR are involved in several important biological events, however early in an infection it would be more important with a triggered innate defence and not a highly specialised antibody response. The polymorphisms in the FcγR will have no influence on the initial infection, unless the parasite has been recognised before and thereby initiated an antibody response. Any new antigenic variants will not primarily be attacked by antibody dependent functions, but rather by innate effector functions. Defects in these primary responses would affect the following development of the immune responses and hence the antibody production. Hence, the effect of polymorphisms in FcγR genes will most likely not affect the primary infection but the following re-infections.

CRP – a potent player in malaria susceptibility?

The acute phase protein CRP raises dramatically during inflammations and infections, and high levels of circulating CRP has been correlated to high malarial parasitemia [161, 162]. Importantly, CRP induces the production of IL-10 [26], which could affect the delicate balance of pro- and anti-inflammatory cytokines that is important to maintain in malaria infections. Moreover this could have an effect on the IgG subclass switch, since IL-10 induces IgG1 and IgG3 in humans [203]. Thus, CRP could affect the antibody levels, and thereby be one reason for the different anti-malarial antibody levels and patterns seen in Fulani and their sympatric ethnic neighbours. Several functional polymorphisms have been reported in the CRP gene [204] although the polymorphisms showing the most promising functional effects on circulating CRP concentrations would be the positions -717, -286, +1444 in the CRP gene [167, 204].

We have been able to show clear ethnic differences in allele frequencies for the CRP -286 C/T/A polymorphism in two independent cohorts in Mali and Sudan (IV), with the A-allele, previously associated with higher CRP levels, being more common in the non-Fulani groups of both countries. The -717 SNP only showed a difference in genotype frequencies in Mali, with the high producing T allele being more common in the non-Fulani group (IV). The +1444 SNP did not show any difference in genotype frequencies in neither Mali nor Sudan (IV). The presence of the two high producing alleles -717 T and the -286 A in the non-Fulani groups suggests that CRP concentrations could be responsible for part of the lower susceptibility to malaria seen in the Fulani ethnic groups.

Can cytokine gene variations be a factor in malaria susceptibility?

Cytokines are important players in the immune response against *Plasmodium* parasites. It has been suggested that a balanced pro- and anti-inflammatory cytokine response is crucial for an efficient clearance of the parasite without an unacceptable pathology [205]. Genetic factors have been postulated to play a role in the differences in the inflammatory response between different individuals. The highly polymorphic promoter regions of IL-1β and TNF have been suggested to contribute to the stable inter-individual differences in inflammatory responses. Moreover, cytokines can affect the acute phase response, and the results we obtained for the CRP gene variants led us to investigate some cytokines related to CRP production. We

analysed polymorphisms in the IL-1β, IL-6, IL-10 and TNF genes in individuals from Mali and Sudan, in order to find a possible explanation to the difference in susceptibility to malaria seen in the Fulani as compared to sympatric neighbours (V).

Only one of the analysed polymorphisms had a similar pattern in genotype frequencies in both Mali and Sudan. The low producing A allele of the -1087 IL10 polymorphism was more common in the Fulani groups than in the non-Fulani groups. This confirmed a previous study in Mali, where this A allele was found to be more prevalent in the Fulani group [67]. Together with the results from the -286 CRP polymorphism (IV), it appears as if Fulani individuals from both Mali and Sudan are not only low producers of CRP, but also of IL-10. This could have a tremendous effect on the anti-inflammatory responses in this ethnic group, and moreover be a possible reason to the lower susceptibility to malaria in this group.

For the Mali groups, significant interethnic differences in genotype frequencies were found in the IL-1β -1464 G/C SNP, with the proposed low-producing C allele being more frequent in the Fulani ethnic group. This polymorphism did not show any interethnic differences in the Sudanese cohort. Instead, the -511 A/G and the -3737 G/A SNPs showed a significant difference in both genotype and allele frequencies, with the low producing -511 A allele being more common in the non-Fulani group, while the low producing -3737 A allele was more frequent in the Fulani group. Also in Mali, there was a tendency for a similar pattern as seen in Sudan regarding the allele frequencies of the -511 polymorphism, although the interethnic differences did not reach statistical significance, the allele associated to low production of IL-1β being more prevalent in the non-Fulani group. Several reports have associated the IL-1β SNP with clinical phenotypes and outcomes of various inflammatory diseases [60], however there are inconsistencies in terms of which specific polymorphic sequence is giving a certain clinical phenotype. It may therefore be a question of intensity of the infection pressure that gives these differences between the Mali and Sudan cohorts. The variations in the promoter region of IL-1β do suggest a possible influence on the susceptibility to malaria, however, rather by a haplotype pattern than by a single polymorphism.

TNF is a cytokine that has been intensively studied in malaria infections, and the main idea for the moment is that TNF is one of the important cytokines that are involved in the clearance of

the parasites, however an excess of TNF may lead to severe pathology [176]. TNF is also a key inducer of the acute phase response. Polymorphisms in the promoter region of the TNF gene (-308, -238, -863, -1031) have been related to different malaria outcomes and severity of the infection [151, 177-181, 206]. The A allele of the -308 TNF SNP did show an ethnic difference in genotype and allele frequencies in Mali, with the A allele more frequent in the non-Fulani group. For our Sudan groups, the -308 did not show any difference, while the -238 A/G did, with the -238 A allele being more common in the non-Fulani group. When comparing asymptomatic individuals to symptomatic patients in Mali, the -308 allele frequency differed in the Fulani groups, with asymptomatic individuals having a lower frequency of the A allele than symptomatic individuals (Israelsson *et al*, unpublished data). The A allele of these two polymorphisms have been related to cerebral malaria and severe malarial anaemia, respectively [180, 181, 206]. The lower frequency of these alleles in the asymptomatic Fulani as compared to non-Fulani individuals and symptomatic Fulani individuals, suggests that this polymorphism might be responsible for some of the lower susceptibility seen in the Fulani in Mali. However, the overall results suggest that a population or individual variation at the TNF locus could affect the susceptibility to malaria, and that probably more than one polymorphism in this area could have this effect.

Haplotypes

Haplotypes are often used as a tool to explore clinical associations with genetic variation over a more or less broad physical region of the genome. This is an efficient way to capture the possible effects of a small number of genetic variants. Haplotypes can also be analysed when a number of polymorphisms in the same region or gene have similar biological effects, and the combination of these polymorphisms could be of importance. These analyses will be more powerful than analyses of each polymorphism alone.

The haplotype analyses for FcγR polymorphisms in relation to ethnicity did not reveal any clear patterns, suggesting that there is no combination of these receptor polymorphisms that will be particularly more powerful in affecting the malaria susceptibility. However, if we had compared the haplotypes among patients with different severity of malaria, the result could probably have been different. Thereby we suggest that the different FcγR polymorphisms do

not affect the initial susceptibility to malaria, but rather affect the actual infection by facilitating a better clearance of the parasites or enhancing the immune responses against the infection.

The FcγRIIa 131 H/R polymorphism has been associated to susceptibility to malaria in several studies [147, 149, 151], and CRP binds with a higher affinity to the receptor expressed by the R allele [153]. The CRP binding might have a competitive effect on the binding of the previously indicated malaria protective IgG1 and IgG3 antibodies [114], thereby interfering with the parasite neutralisation. Since the *CRP* gene and the *FcγR* genes are located in the same region on chromosome 1 (fig. 18), we investigated the possibility of a susceptibility locus on this chromosome.

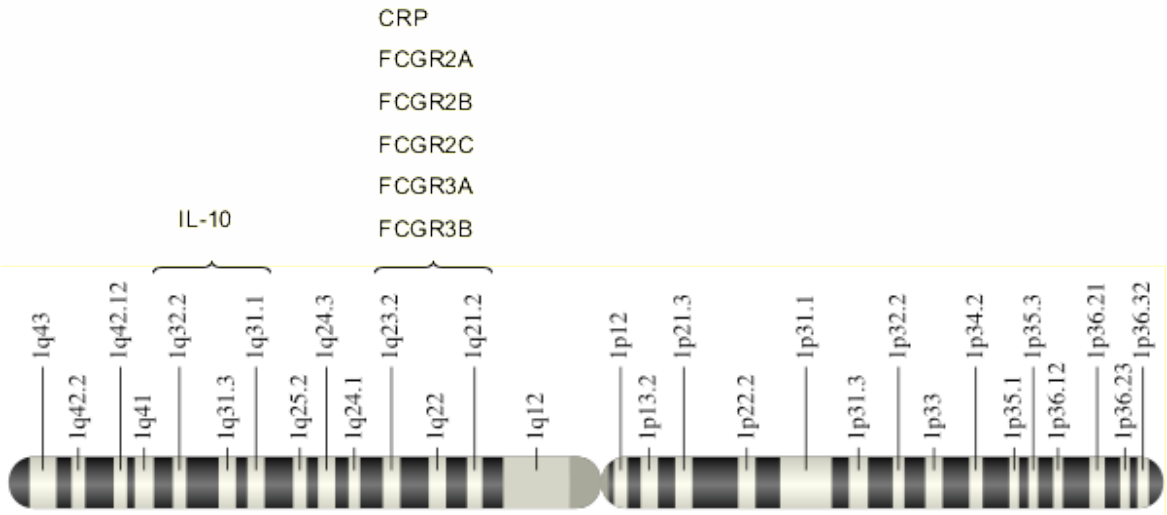


Figure 18. The location of the genes coding for CRP, Fcγ receptors and IL-10 on chromosome 1

Although the linkage analysis did not show any linkage between the CRP -286 and FcγRIIa 131 polymorphisms, the haplotype analyses, interestingly showed associations between haplotypes including the CRP -286 A allele and the non-Fulani groups. This suggests that the -286 CRP polymorphism might be a genetic factor that could be of high relevance in malaria susceptibility. Furthermore, since both the alleles of the FcγRIIa 131 R/H are found in haplotypes associated with both Fulani and non-Fulani, and the CRP-286 C>T>A alleles are restricted to only one ethnic group, it may be the CRP genotype rather than the FcγRIIa allotype that confers the relative protection from malaria seen in the Fulani ethnic groups.

The analyses of the cytokine polymorphisms revealed a possible influence of the different alleles on the relative susceptibility to malaria. Haplotype analyses of the polymorphic variants within each gene did show a difference in haplotype patterns between Fulani and

non-Fulani individuals in both Mali and Sudan, with no major differences between the two countries. In general, there was a larger variety of haplotypes in Sudan, but the most prevalent haplotypes were the same in both countries, although at different frequencies. The main finding being consistent in both Mali and Sudan were regarding the IL-1β high-producing haplotype GGG/GGA (higher IL-1β), which were more prevalent in the Fulani groups in both countries. Moreover, the IL-10 high-producing haplotype GCC/GCC was more prevalent in the non-Fulani groups, while the low-producing haplotype ATA/ATA was found to be more frequent in the Sudanese Fulani group. All differences in haplotype frequency between asymptomatic and symptomatic individuals were seen only in the Fulani group. Interestingly, the haplotypes for the IL-1β and IL-10 polymorphisms that showed a difference between symptomatic and asymptomatic individuals also differed between asymptomatic Fulani and non-Fulani individuals (Israelsson *et al* unpublished data), suggesting that these cytokine haplotypes could have an influence on the susceptibility to malaria. The TNF haplotype TCGG/TCGG did not differ between asymptomatic Fulani and non-Fulani, although found in a higher frequency in the symptomatic Fulani individuals. This haplotype has the high-producing alleles of all the included polymorphisms, which supports the previous studies linking high TNF levels to severe malaria. Moreover, this finding suggests that TNF is involved in the control of the parasitemia and/or of the infection, but does not exert any influence on the susceptibility to the actual infection. IL-6 haplotypes did not show any significant differences in frequency between asymptomatic Fulani and non-Fulani. However, as for TNF, one haplotype showed a difference between symptomatic and asymptomatic Fulani individuals, suggesting that IL-6 is involved in clearance of the infection or the protection against the disease, but not in the interethnic differences in the susceptibility to malaria seen in the Fulani and non-Fulani ethnic groups.

Biological associations of the genetic variations

If a genetic variant or haplotype lacks relevant biological associations, it will be very difficult to evaluate its role in a disease. Parasitemia and haemoglobin levels are two well-characterised factors related to the severity of a malaria infection. The number of *msp-2* clones was lower in H-allele carriers of the FcγRIIa R131H polymorphism than RR homozygotes in the Fulani group, while individuals homozygous for the H-allele in the Dogon group had fewer parasite positive individuals than the HR and RR genotypes carriers (II). Thus, these data suggest a protective effect of the H-allele or the HH genotype, which is

supported by the results of other studies [149, 151, 152]. An additional association with the haemoglobin levels was seen for the FcγRIIb T232I polymorphism (III), where individuals with the II allotype had lower Hb. These results are in line with the study performed by Clatworthy *et al*, demonstrating that FcγRIIb deficient mice are less anaemic and have lower parasitemia than wild-type mice [155]. In addition, we showed that parasite positive individuals had more often the -286 CRP A-allele than parasite negative individuals, suggesting an increase in susceptibility to malaria if the A-allele is present. However, no difference was seen for the -717 CRP polymorphism regarding parasitemia, which further emphasises the potential importance of the -286 CRP polymorphism in this context (IV).

Our finding that the R-allele of the FcγRIIa R131H polymorphism showed an association with the IgG3 antibody levels in both the Fulani and the Dogon further strengthened the suggestion of a possible influence of this polymorphism on IgG subclass profile (II). Also, we could see a trend for an association of the HH genotype with the total IgG1 levels, and it appears as if also the IgG2 levels could be influenced by this FcγRIIa polymorphism. However, the results were inconclusive and contradictory in the two ethnic groups, with Fulani HH carriers having higher IgG2 antibody levels, while the RR carriers had higher levels in the Dogon (II). A study by Scopel *et al* [207] investigated the association of the FcγRIIa 131 R/H polymorphism with the antibody responses against the *P. falciparum* antigens MSP-1 and MSP-2, but no associations between FcγRIIa allotype and IgG subclass pattern were observed. However, MSP-1 and -2 are giving strong IgG1 and IgG3 responses, which could disguise an influence of the FcγRIIa allotype. In a recent study, Leoratti *et al* [152] confirmed our results regarding the association of the FcγRIIa H allele with IgG2 antibody levels. Leoratti et al used a *P. falciparum* blood stage antigen, similar to our crude *P. falciparum* extract, suggesting that the possible influence of the FcγRIIa allotypes can be seen on the levels of IgG2 antibodies to crude antigen preparations as well as on total IgG subclass levels.

We observed differences in CRP concentrations between the different -717 and -286 CRP genotypes, which also was shown in previous studies [167, 204]. However, no associations of CRP levels with any of the cytokine haplotypes were detected.

Concluding remarks

The aim of this thesis was to elucidate some of the possible pathways that control antibody responses in malaria infected individuals. The genetic approach used in the studies discussed above have helped in shedding some light over the ethnic differences in the susceptibility to malaria between the Fulani and other ethnicities.

This thesis has showed possible connections between different cytokines, CRP, FcγR, Ig class switch and the following antibody responses. A connection between FcγRIIa R131H and IgG subclass patterns were shown in paper II. Paper IV showed that the Fulani are genetically predisposed to have a lower production of CRP, and an association between the functional polymorphism -286 and parasitemia. Paper V showed that the Fulani have a higher frequency of the low producing IL-10 and the high producing IL-1β haplotypes, and a lower frequency of the alleles in TNF previously associated to severe malaria. Moreover, in paper II we could show that the IgG subclass patterns differ between the two ethnic groups.

In an individual with a single malaria infection, inflammatory responses will be rapid, with the up regulation of IL-1β, TNF, IL-6, IFN-γ and CRP, which will lead to a control of the parasitemia. IL-6 will soon control its own production by the up-regulation of soluble receptors, and the increasing concentrations of CRP will induce the production of IL-10. The up-regulation of IL-10 by the cells, will lead to a lower control of the parasitemia, due to the anti-inflammatory action of IL-10, but also to a reduction in the symptoms, as inflammation increases the severity of the symptoms. In all, this balanced response will clear the infection with minimal symptoms. However, in an area with endemic malaria, the risk of having multiple, overlapping or repeated infections is high. In this scenario, the increased anti-inflammatory response in the end of the infection could have an inhibiting effect also on the inflammatory response mounted to the new infection. If so, this will lead to decreased control of the parasitemia, but with limited symptoms. This might be one explanation to the asymptomatic infections with high parasitemia that can be seen in a few individuals in endemic countries. Our results suggest that the Fulani ethnic groups have higher inflammatory responses and lower anti-inflammatory responses as compared to the sympatric ethnic groups. This could be due to a selection for a more beneficial phenotype in individuals

living with endemic malaria; the ability to mount an inflammatory response despite an anti-inflammatory milieu, in the end leads to an efficient clearance of the infections. Together with different FcγR haplotypes, this could have a visible effect on IgG concentrations for some infections. The end-result would then be a more activated phenotype in the Fulani (fig. 19A) and a more inhibitory/anti-inflammatory phenotype in the non-Fulani (fig.19B).

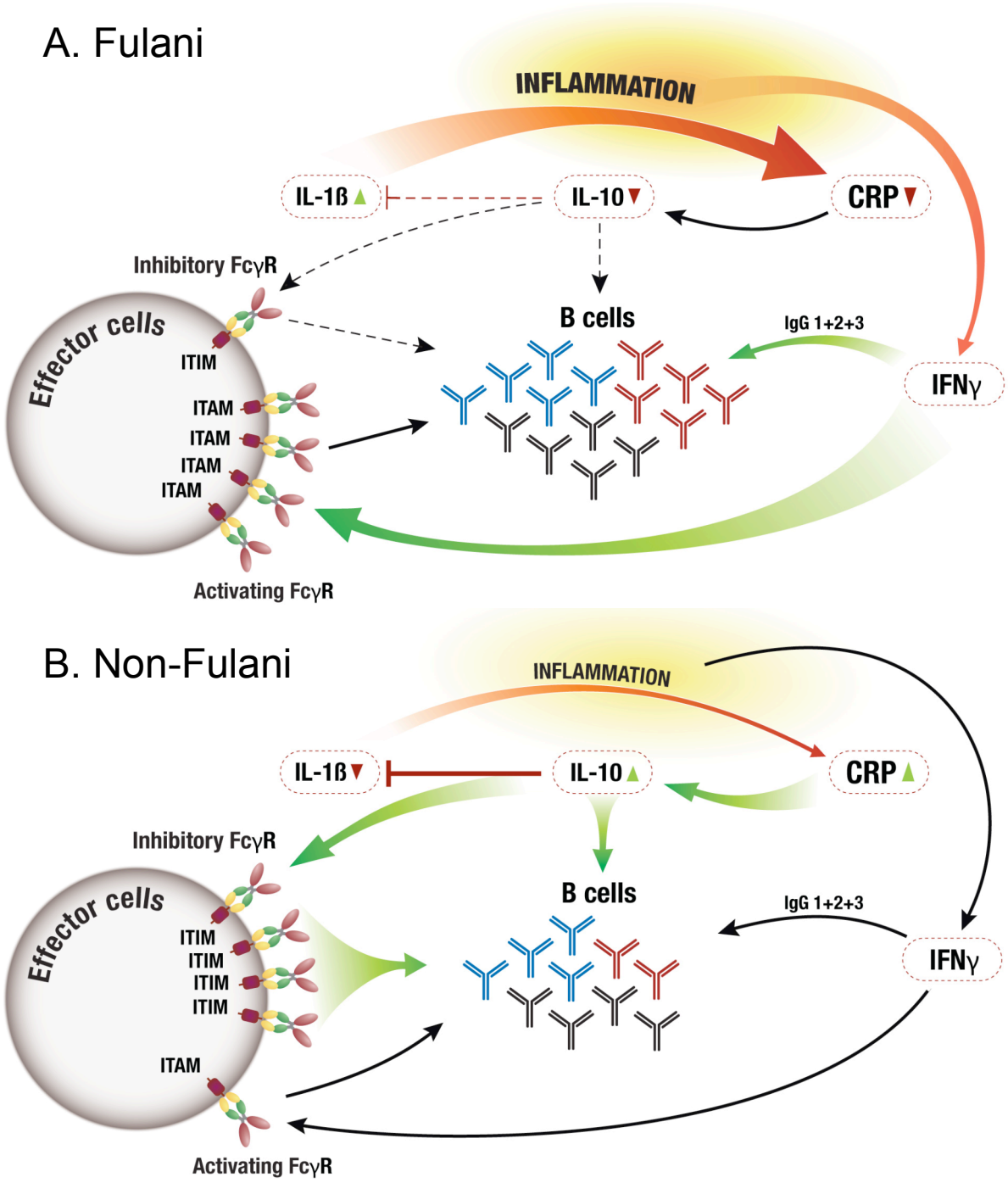


Figure 19. A: The predicted higher production of IL-1β in the Fulani ethnic group will mount a strong and fast inflammatory response. This will lead to activation of CRP in the hepatocytes, and this increase in CRP will later induce IL-10 that will inhibit the production of IL-1β. However, the predicted low production of IL-10 in the Fulani will not be strong enough to limit the inflammation completely. Moreover, IL-10 will induce the production of IgG1 and IgG3 as well as increase the expression of inhibitory FcγR. These effects will not be as strong in the Fulani as compared to the non-Fulani. The inflammatory response will increase the production of IFN-γ, which will induce the production of IgG1, IgG2 and IgG3 as well as the expression of activating FcγR. This will have an effect on the antibody pool. **B:** The lower/normal production of IL-1β in the non-Fulani will mount a normal inflammation, however, being high producers of both CRP and IL-10, the non-Fulani will have a stronger effect of the anti-inflammatory IL-10 than the Fulani, hence non-Fulani will present a higher frequency of inhibitory FcγR and lower antibody levels. Moreover, the inflammation in the non-Fulani will not be as pronounced as in the Fulani, resulting in fewer activating FcγR and a lower production of IgG2.

Future prospects

The studies in this thesis have revealed an interesting connection between innate functions and adaptive immune functions. The future prospects would include further studies of interplay between these aspects. The possible up-regulation of inhibitory FcγR in the non-Fulani group, together with the results from Torcia *et al* [174], which shown a functional deficit of Tregs in the Fulani, could suggest a role of the regulatory mechanisms in the susceptibility to malaria. The reduced population of possible regulatory B-cells in MS patients could be an interesting path to follow [32]. Regulatory B-cells could have the capacity to affect the antibody production, and a similar functional deficit in this cell population as shown for Tregs, would have tremendous effects on the immune responses to several infections.

Moreover, IL-17 is a cytokine with the capacity to influence the levels of CRP. It orchestrate the innate immune functions and it plays a central role in autoimmune inflammations, but is at the same time essential in the protection from infections, by recruiting neutrophils to the infected tissues [208]. No studies have, to our knowledge, yet showed a role for IL-17 in malaria. However, in experimental murine *S. mansoni* infections, IL-17 has been associated to a severe pathology [209, 210].

In addition, functional experiments with the different FcγR allotypes, the effect of the cytokine haplotypes on the circulating levels of the cytokines, and functional experiments with CRP will also be important to investigate in order to further elucidate the impact of these factors on the IgG subclass pattern, and the susceptibility to malaria.

Acknowledgements

Behind every student stand a supervisor, colleagues and others. I managed to go through these years without to many difficulties, but I know that I couldn't have done this alone. I would like to express my sincere gratitude to everyone that have contributed in one way or another in making this thesis a reality.

I am very grateful for all the help and support I got from my supervisor, **Klavs Berzins**. You never held me back, even though some of my ideas were very expensive and time consuming, and you always took your time to hear what I had to say. You helped me become an independent thinker who is not afraid of questioning "truths" and facts. But I think the most amazing thing you did was to send me around the world on conferences, courses and fieldwork, and yet managed to make me finish on time!

All the seniors at the department, **Carmen, Marita, Eva** and **Eva** have all been really inspiring and always ready to answer my questions. **Marita**, you have always, even though not being my official supervisor, been very interested in my work and always had many ideas and thoughts about the results.

Maggan, Ann, Gelana and **Anna-Leena** are the foundation that the whole Immunology department relies on. All small, but crucial details, are being organized by you and it is really noticed when you are on holidays. You are always ready to help and you all do it with a smile. Keep up the good work and always remember that you are invaluable!

As every student soon finds out, nothing is unbearable with good friends around. I am very glad to have got to know all of you: **the magnificent malaria group, the amazing allergy girls and the terrific TB boys**! I especially want to acknowledge **Yvonne**, who was a good support during the last months; **Petra**, who not only inspired me more than anyone else, but also made me understand that life is meant to be lived; **Pablo**, whose wit, humour and relaxed outlook on life is outstanding and who encouraged me whenever I hesitated; **Halima** who taught me that everything is possible as long as you really want it and **Manijeh**, a great travel companion and friend who is always ready to help.

All the undergrads that have been helping me in the different projects, **Anna Lysén, Gishanthi Arambepola** and **Susannah Kearlsey**. Your input has been invaluable and I'm really thankful for all your help. Without you, I would probably not have had this amount of interesting results!

Även om många inom min familj och vän krets knappt förstår vad jag har hållit på med (jobba dag och natt och vissa perioder vägra svara i telefonen, åka till Mali för att sticka folk i fingret och kladda en bloddroppe på en pappersbit, njuta av att inte komma åt sin e-mail, rationalisera bort socialt umgämge för att skriva på artiklar som ändå inte publiceras i någon tidning som man känner till, osv) så har jag alltid blivit uppmuntrad och ivrigt påhejad.

Jag minns hur en vittfrämmande tjej första dagen på genetik kursen utan att röra en min svarar på min fråga om vem hon labbar med: "Med dig". Det var starten på en ovärderlig vänskap. Min **Jenny**. Du får mig alltid på bra humör, du ställer upp på många upptåg och infall och du har dessutom fört in **Johanna** i mitt liv. Vad skulle jag ha gjort utan er? Vi var alla på samma punkt i livet: doktorander som försökte få någon rätsida på allt. Jag tror att jag skulle ha blivit galen om det inte vore för våra ventileringsträffar. Jag är så tacksam för vår vänskap och jag vet att vi har många roliga episoder kvar att uppleva tillsammans.

Hasse, Maria, Ludde och **Chandler**, det är väl ingen hemlighet att jag är förtjust i Chandler så hon kan lika gärna få stå med här hon också ☺ Tack för alla middagar, skidresor, midsommarfester och annat skoj som ni ordnat! Tack Chandler för att jag får begrava näsan i din underbara kattpäls då och då! Och jag får inte glömma **Svante** och **Sixten** heller, trots allt är de lika gosiga dom och Svante brukar ju så vänligt städa upp på mitt köksgolv varje gång han är på besök! ☺. Tack även till Hasse som så glatt gick med på att vara toastmaster!

Palle och **Frida**, inte nog med alla roliga kvällar ni bjuder på, ni har även hjälpt till med allt möjligt inför min disputation. Palle är dessutom begåvningen som gjort illustrationerna i den här avhandlingen. Tack till er bägge!

Tack till **Eva** långt-bort-i-Ramnäs, för att du är en sån underbar person och att jag fått följa med på härliga ridturer i Ramnässkogarna!

Till alla mina vänner i **Capoeira Raízes** (ingen nämnd och ingen glömd)**.** När jag började med capoeira trodde jag inte att jag skulle få så många vänner med på köpet, men det fick jag. Det har varit väldigt kul att få lära känna så många olika typer av människor, på olika ställen i livet, med så olika mål. Ni är alltid redo för att ha kul och ni vet hur man roar sig! Muito obrigada por tudo!

Det finns en enda sak som jag inte klarar mig utan och det är hästar. Jag försökte under flera år, men fick till slut ett återfall. Och vilket återfall sen! Zelzele, ett stort vackert fuxsto, som har bundit ihop människor som aldrig troligtvis skulle ha träffats. **Stall Zygot**, innehållandes Leif, Peter, Elisabeth, Fred, Marita, Bertil och Petra har gjort det möjligt för mig att ha en häst i mitt liv igen. Att hon sedan kanske kommer att springa in lite pengar då och då gör ju inte saken sämre! ☺

Familjen Fernstad, med Eunice, Roland, Josefin och Jonatan, tack för allt stöd ni har gett mig och ert uppriktiga intresse för mitt arbete!

Min underbara familj, mamma Birgitta, pappa Paul och syster Kristin. Tack för allt stöd och kärlek ni har gett mig. Ni har stått ut med det mesta under dessa år, utan att klaga. Jag är övertygad att jag inte skulle ha kunnat göra det här utan er! **Nina**, min alldeles egna stora-syster. Du kommer alltid, oavsett var jag befinner mig, vara Stora Syster-Yster och jag kommer att vara din Lill-Strumpa.

Rikard och Amalia, mina underbara syskonbarn. Jag hoppas att ni fortsätter uppleva allt spännande som livet och naturen erbjuder och att ni alltid vågar testa era gränser. För det är då som det händer magiska ting!

Min älskade Joakim, du är mitt stöd, mitt äventyr, mitt liv. Utan din uppmuntran och problemlösning hade detta inte gått.

References

1. Wickelgren I. Immunology. Targeting the tolls. *Science* 2006; 312: 184-187

2. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005; 17: 1-14

3. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; 5: 987-995

4. Meyer-Bahlburg A, Khim S, Rawlings DJ. B cell intrinsic TLR signals amplify but are not required for humoral immunity. *J Exp Med* 2007; 204: 3095-3101

5. Xu D, Komai-Koma M, Liew FY. Expression and function of Toll-like receptor on T cells. *Cell Immunol* 2005; 233: 85-89

6. Whiteside TL, Herberman RB. Role of human natural killer cells in health and disease. *Clin Diagn Lab Immunol* 1994; 1: 125-133.

7. Larosa DF, Orange JS. 1. Lymphocytes. *J Allergy Clin Immunol* 2008; 121: S364-9; quiz S412

8. Vivier E*, et al*. Functions of natural killer cells. *Nat Immunol* 2008; 9: 503-510

9. O'Leary JG*, et al*. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* 2006; 7: 507-516

10. Zhang C, Zhang J, Tian Z. The regulatory effect of natural killer cells: do "NK-reg cells" exist? *Cell Mol Immunol* 2006; 3: 241-254.

11. Gratchev A*, et al*. MΦ1 and MΦ2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology* 2006; 211: 473-486

12. Reschner A*, et al*. Innate lymphocyte and dendritic cell cross-talk: a key factor in the regulation of the immune response. *Clin Exp Immunol* 2008; 152: 219-226

13. Ullrich E, Chaput N, Zitvogel L. Killer dendritic cells and their potential role in immunotherapy. *Horm Metab Res* 2008; 40: 75-81

14. Parker LC*, et al*. The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J Leukoc Biol* 2005; 77: 886-892

15. Prussin C, Metcalfe DD. 5. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* 2006; 117: S450-6

16. Wang HB*, et al*. Airway eosinophils: allergic inflammation recruited professional antigen-presenting cells. *J Immunol* 2007; 179: 7585-7592.

17. Lu LF*, et al*. Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 2006; 442: 997-1002

18. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 1999; 340: 448-454.

19. Baumann H, Gauldie J. The acute phase response. *Immunol Today* 1994; 15: 74-80.

20. Patel DN, *et al*. Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-κB and C/EBPbeta activation. *J Biol Chem* 2007; 282: 27229-27238

21. Volanakis JE, Kaplan MH. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proc Soc Exp Biol Med* 1971; 136: 612-614.

22. Bodman-Smith KB, *et al*. C-reactive protein-mediated phagocytosis of *Leishmania donovani* promastigotes does not alter parasite survival or macrophage responses. *Parasite Immunol* 2002; 24: 447-454.

23. Casey R, *et al*. The acute-phase reactant C-reactive protein binds to phosphorylcholine-expressing *Neisseria meningitidis* and increases uptake by human phagocytes. *Infect Immun* 2008; 76: 1298-1304

24. Weiser JN, *et al*. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J Exp Med* 1998; 187: 631-640.

25. Du Clos TW, Mold C. C-reactive protein: an activator of innate immunity and a modulator of adaptive immunity. *Immunol Res* 2004; 30: 261-77.

26. Marnell L, Mold C, Du Clos TW. C-reactive protein: ligands, receptors and role in inflammation. *Clin Immunol* 2005; 117: 104-11.

27. Bang R, *et al*. Analysis of binding sites in human C-reactive protein for FcγRI, FcγRIIA, and C1q by site-directed mutagenesis. *J Biol Chem* 2005; 280: 25095-25102

28. Zhang R, *et al*. C-reactive protein impairs human CD14+ monocyte-derived dendritic cell differentiation, maturation and function. *Eur J Immunol* 2006; 36: 2993-3006

29. Zhong W, *et al*. Effect of human C-reactive protein on chemokine and chemotactic factor-induced neutrophil chemotaxis and signaling. *J Immunol* 1998; 161: 2533-2540.

30. Zahedi K, Mortensen RF. Macrophage tumoricidal activity induced by human C-reactive protein. *Cancer Res* 1986; 46: 5077-5083.

31. Nazmi A, Victora CG. Socioeconomic and racial/ethnic differentials of C-reactive protein levels: a systematic review of population-based studies. *BMC Public Health* 2007; 7: 212

32. Mauri C, Ehrenstein MR. The 'short' history of regulatory B cells. *Trends Immunol* 2008; 29: 34-40

33. Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev* 2003; 8: 223-246.

34. Bettelli E, *et al*. Induction and effector functions of $T_{(H)}17$ cells. *Nature* 2008; 453: 1051-1057

35. Cools N, *et al*. Regulatory T cells and human disease. *Clin Dev Immunol* 2007; 2007: 89195

36. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* 2004; 114: 1379-1388

37. Chien YH, Jores R, Crowley MP. Recognition by γ/δ T cells. *Annu Rev Immunol* 1996; 14: 511-532

38. Brandes M, Willimann K, Moser B. Professional antigen-presentation function by human γδ T Cells. *Science* 2005; 309: 264-268

39. Harding CV, Unanue ER. Cellular mechanisms of antigen processing and the function of class I and II major histocompatibility complex molecules. *Cell Regul* 1990; 1: 499-509.

40. Martin NH. The immunoglobulins: a review. *J Clin Pathol* 1969; 22: 117-131.

41. Vollmers HP, Brandlein S. Natural IgM antibodies: the orphaned molecules in immune surveillance. *Adv Drug Deliv Rev* 2006; 58: 755-765

42. Hjelm F*, et al*. Antibody-mediated regulation of the immune response. *Scand J Immunol* 2006; 64: 177-184

43. Vladutiu AO. Immunoglobulin D: properties, measurement, and clinical relevance. *Clin Diagn Lab Immunol* 2000; 7: 131-140.

44. Pan Q, Hammarstrom L. Molecular basis of IgG subclass deficiency. *Immunol Rev* 2000; 178: 99-110.

45. Kerr MA. The structure and function of human IgA. *Biochem J* 1990; 271: 285-296.

46. Thapa BR. Health factors in colostrum. *Indian J Pediatr* 2005; 72: 579-581.

47. Heyman B. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu Rev Immunol* 2000; 18: 709-737

48. Estambale BB*, et al*. *Bancroftian filariasis* in Kwale District of Kenya. III. Quantification of the IgE response in selected individuals from an endemic community. *Ann Trop Med Parasitol* 1995; 89: 287-295.

49. Ramirez RM*, et al*. The immunopathology of human *Schistosomiasis*-III. Immunoglobulin isotype profiles and response to praziquantel. *Mem Inst Oswaldo Cruz* 1996; 91: 593-599.

50. Rossi CL*, et al*. Total serum IgE and parasite-specific IgG and IgA antibodies in human strongyloidiasis. *Rev Inst Med Trop Sao Paulo* 1993; 35: 361-365.

51. Perlmann H*, et al*. IgE elevation and IgE anti-malarial antibodies in *Plasmodium falciparum* malaria: association of high IgE levels with cerebral malaria. *Clin Exp Immunol* 1994; 97: 284-92.

52. Perlmann P*, et al*. IgE and tumor necrosis factor in malaria infection. *Immunol Lett* 1999; 65: 29-33.

53. Calissano C*, et al*. IgE antibodies to *Plasmodium falciparum* and severity of malaria in children of one ethnic group living in Burkina Faso. *Am J Trop Med Hyg* 2003; 69: 31-5.

54. Bereczky S*, et al*. Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria. *Int J Parasitol* 2004; 34: 935-42.

55. Daeron M. Fc receptor biology. *Annu Rev Immunol* 1997; 15: 203-234

56. Pleass RJ, Woof JM. Fc receptors and immunity to parasites. *Trends Parasitol* 2001; 17: 545-551

57. Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. *Nat Rev Immunol* 2008; 8: 34-47

58. Shi J, McIntosh RS, Pleass RJ. Antibody- and Fc-receptor-based therapeutics for malaria. *Clin Sci (Lond)* 2006; 110: 11-19

59. Dinarello CA. The interleukin-1 family: 10 years of discovery. *FASEB J* 1994; 8: 1314-1325.

60. Rogus J*, et al*. IL1β gene promoter haplotype pairs predict clinical levels of interleukin-1β and C-reactive protein. *Hum Genet* 2008; 123: 387-398

61. Kishimoto T. Interleukin-6: discovery of a pleiotropic cytokine. *Arthritis Res Ther* 2006; 8 Suppl 2: S2

62. Rose-John S. Interleukin-6 biology is coordinated by membrane bound and soluble receptors. *Acta Biochim Pol* 2003; 50: 603-611

63. Jones SA*, et al*. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *FASEB J* 2001; 15: 43-58

64. O'Garra A, Vieira P. $T_{(H)}1$ cells control themselves by producing interleukin-10. *Nat Rev Immunol* 2007; 7: 425-428

65. van Lent PL*, et al*. Toll-like receptor 4 induced FcγR expression potentiates early onset of joint inflammation and cartilage destruction during immune complex arthritis: Toll-like receptor 4 largely regulates FcγR expression by interleukin 10. *Ann Rheum Dis* 2007; 66: 334-340

66. Wilczynski JR, Radwan M, Kalinka J. The characterization and role of regulatory T cells in immune reactions. *Front Biosci* 2008; 13: 2266-2274.

67. Vafa M*, et al*. Associations between the IL-4 -590 T allele and *Plasmodium falciparum* infection prevalence in asymptomatic Fulani of Mali. *Microbes Infect* 2007; 9: 1043-1048

68. Pachkoria K*, et al*. Analysis of IL-10, IL-4 and TNF-alpha polymorphisms in drug-induced liver injury (DILI) and its outcome. *J Hepatol* 2008; 49: 107-114

69. Bradley JR. TNF-mediated inflammatory disease. *J Pathol* 2008; 214: 149-160

70. Arenzana-Seisdedos F, Virelizier JL, Fiers W. Interferons as macrophage-activating factors. III. Preferential effects of interferon-gamma on the interleukin 1 secretory potential of fresh or aged human monocytes. *J Immunol* 1985; 134: 2444-2448.

71. Winkler S*, et al*. Reciprocal regulation of Th1- and Th2-cytokine-producing T cells during clearance of parasitemia in *Plasmodium falciparum* malaria. *Infect Immun* 1998; 66: 6040-6044.

72. Luty AJ*, et al*. Interferon-gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *J Infect Dis* 1999; 179: 980-988

73. Poinar G,Jr. *Plasmodium dominicana n. sp.* (*Plasmodiidae: Haemospororida*) from Tertiary Dominican amber. *Syst Parasitol* 2005; 61: 47-52

74. Joy DA*, et al*. Early origin and recent expansion of *Plasmodium falciparum*. *Science* 2003; 300: 318-321

75. Cox FE. History of human parasitology. *Clin Microbiol Rev* 2002; 15: 595-612.

76. Weina PJ. Artemisinins from folklore to modern medicine--transforming an herbal extract to life-saving drugs. *Parassitologia* 2008; 50: 25-29.

77. Bradbury J. Malaria sensitivity to chloroquine returns to Malawi. *Lancet Infect Dis* 2007; 7: 11.

78. Suh KN, Kain KC, Keystone JS. Malaria. *CMAJ* 2004; 170: 1693-1702.

79. Cox-Singh J*, et al*. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* 2008; 46: 165-171

80. Cogswell FB. The hypnozoite and relapse in primate malaria. *Clin Microbiol Rev* 1992; 5: 26-35.

81. Kirchgatter K, Del Portillo HA. Clinical and molecular aspects of severe malaria. *An Acad Bras Cienc* 2005; 77: 455-475

82. Doolan DL, Martinez-Alier N. Immune response to pre-erythrocytic stages of malaria parasites. *Curr Mol Med* 2006; 6: 169-185.

83. McGilvray ID*, et al*. Nonopsonic monocyte/macrophage phagocytosis of *Plasmodium falciparum*-parasitized erythrocytes: a role for CD36 in malarial clearance. *Blood* 2000; 96: 3231-3240.

84. Jones KR*, et al*. Killing of *Plasmodium falciparum* by human monocyte-derived macrophages. *Parasite Immunol* 1989; 11: 585-592.

85. Orago AS, Facer CA. Cytotoxicity of human natural killer (NK) cell subsets for *Plasmodium falciparum* erythrocytic schizonts: stimulation by cytokines and inhibition by neomycin. *Clin Exp Immunol* 1991; 86: 22-29.

86. Serghides L*, et al*. CD36 and malaria: friends or foes? *Trends Parasitol* 2003; 19: 461-469

87. Dodoo D*, et al*. Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *Plasmodium falciparum* malaria. *J Infect Dis* 2002; 185: 971-979

88. Artavanis-Tsakonas K, Riley EM. Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. *J Immunol* 2002; 169: 2956-2963.

89. Hansen DS*, et al*. CD1d-restricted NKT cells contribute to malarial splenomegaly and enhance parasite-specific antibody responses. *Eur J Immunol* 2003; 33: 2588-2598

90. Urban BC*, et al*. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* 1999; 400: 73-77

91. Urban BC, Roberts DJ. Inhibition of T cell function during malaria: implications for immunology and vaccinology. *J Exp Med* 2003; 197: 137-141.

92. Perry JA*, et al*. Dendritic cells from malaria-infected mice are fully functional APC. *J Immunol* 2004; 172: 475-482.

93. Wykes MN*, et al*. *Plasmodium* strain determines dendritic cell function essential for survival from malaria. *PLoS Pathog* 2007; 3: e96

94. Farouk SE*, et al*. Gamma delta T cells inhibit in vitro growth of the asexual blood stages of *Plasmodium falciparum* by a granule exocytosis-dependent cytotoxic pathway that requires granulysin. *Eur J Immunol* 2004; 34: 2248-2256

95. Nardin EH, Nussenzweig RS. T cell responses to pre-erythrocytic stages of malaria: role in protection and vaccine development against pre-erythrocytic stages. *Annu Rev Immunol* 1993; 11: 687-727

96. Hill AV*, et al*. Common west African HLA antigens are associated with protection from severe malaria. *Nature* 1991; 352: 595-600

97. Aidoo M, Udhayakumar V. Field studies of cytotoxic T lymphocytes in malaria infections: implications for malaria vaccine development. *Parasitol Today* 2000; 16: 50-56

98. Stephens R, Langhorne J. Priming of CD4+ T cells and development of CD4+ T cell memory; lessons for malaria. *Parasite Immunol* 2006; 28: 25-30

99. Noben-Trauth N. Susceptibility to *Leishmania major* infection in the absence of IL-4. *Immunol Lett* 2000; 75: 41-44

100. Schofield L, Grau GE. Immunological processes in malaria pathogenesis. *Nat Rev Immunol* 2005; 5: 722-735

101. Luoni G*, et al*. Antimalarial antibody levels and IL4 polymorphism in the Fulani of West Africa. *Genes Immun* 2001; 2: 411-414

102. Riley EM*, et al*. Regulating immunity to malaria. *Parasite Immunol* 2006; 28: 35-49

103. Krishnegowda G*, et al*. Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J Biol Chem* 2005; 280: 8606-8616

104. Coban C, et al. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med 2005; 201: 19-25

105. Parroche P, et al. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci U S A 2007; 104: 1919-1924.

106. Mockenhaupt FP, et al. Toll-like receptor (TLR) polymorphisms in African children: Common TLR-4 variants predispose to severe malaria. Proc Natl Acad Sci U S A 2006; 103: 177-182

107. Mockenhaupt FP, et al. Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy. J Infect Dis 2006; 194: 184-188

108. Cohen S, McGregor IA, Carrington S. Gamma-globulin and acquired immunity to human malaria. Nature 1961; 192: 733-737.

109. Bouharoun-Tayoun H, et al. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J Exp Med 1990; 172: 1633-41.

110. Bolad A, Berzins K. Antigenic diversity of *Plasmodium falciparum* and antibody-mediated parasite neutralization. Scand J Immunol 2000; 52: 233-9.

111. Shi YP, et al. Fcγ receptor IIa (CD32) polymorphism is associated with protection of infants against high-density *Plasmodium falciparum* infection. VII. Asembo Bay Cohort Project. J Infect Dis 2001; 184: 107-11.

112. Tebo AE, Kremsner PG, Luty AJ. *Plasmodium falciparum*: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro. Exp Parasitol 2001; 98: 20-28

113. Gysin J, et al. In vitro phagocytosis inhibition assay for the screening of potential candidate antigens for sub-unit vaccines against the asexual blood stage of *Plasmodium falciparum*. J Immunol Methods 1993; 159: 209-219.

114. Aribot G, et al. Pattern of immunoglobulin isotype response to *Plasmodium falciparum* blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa). Am J Trop Med Hyg 1996; 54: 449-57.

115. Taylor RR, et al. IgG3 antibodies to *Plasmodium falciparum* merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. Am J Trop Med Hyg 1998; 58: 406-13.

116. Egan AF, et al. Serum antibodies from malaria-exposed people recognize conserved epitopes formed by the two epidermal growth factor motifs of MSP1(19), the carboxy-terminal fragment of the major merozoite surface protein of *Plasmodium falciparum*. Infect Immun 1995; 63: 456-66.

117. Pleass RJ, Holder AA. Opinion: antibody-based therapies for malaria. Nat Rev Microbiol 2005; 3: 893-899

118. Berzins K, Anders R. The Malaria antigens. In: Wahlgren M, Perlmann P, eds. Malaria, Molecular and clinical aspects. Amsterdam: Harwood academic publisher, 1999, pp. 181-216.

119. Winter G, et al. SURFIN is a polymorphic antigen expressed on *Plasmodium falciparum* merozoites and infected erythrocytes. J Exp Med 2005; 201: 1853-1863

120. Ahlborg N, et al. *Plasmodium falciparum*: differential parasite growth inhibition mediated by antibodies to the antigens Pf332 and Pf155/RESA. Exp Parasitol 1996; 82: 155-63.

121. Perrin LH, Dayal R. Immunity to asexual erythrocytic stages of *Plasmodium falciparum*: role of defined antigens in the humoral response. Immunol Rev 1982; 61: 245-269.

122. Sharma P, et al. Characterization of protective epitopes in a highly conserved *Plasmodium falciparum* antigenic protein containing repeats of acidic and basic residues. Infect Immun 1998; 66: 2895-2904.

123. Banyal HS, Inselburg J. Isolation and characterization of parasite-inhibitory *Plasmodium falciparum* monoclonal antibodies. Am J Trop Med Hyg 1985; 34: 1055-1064.

124. Hommel M, David PH, Oligino LD. Surface alterations of erythrocytes in *Plasmodium falciparum* malaria. Antigenic variation, antigenic diversity, and the role of the spleen. J Exp Med 1983; 157: 1137-1148.

125. Hviid L. Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. Acta Trop 2005; 95: 270-275

126. Salanti A, et al. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med 2004; 200: 1197-1203

127. Wipasa J, et al. Immunity to asexual blood stage malaria and vaccine approaches. Immunol Cell Biol 2002; 80: 401-414.

128. Mackinnon MJ, et al. Heritability of malaria in Africa. PLoS Med 2005; 2: e340

129. Butcher G. Autoimmunity and malaria. Trends Parasitol 2008

130. Sotgiu S, et al. Multiple sclerosis and anti-*Plasmodium falciparum* innate immune response. J Neuroimmunol 2007; 185: 201-207

131. Sotgiu S, et al. Hygiene hypothesis: innate immunity, malaria and multiple sclerosis. Med Hypotheses 2008; 70: 819-825

132. Terrenato L, et al. Decreased malaria morbidity in the Tharu people compared to sympatric populations in Nepal. Ann Trop Med Parasitol 1988; 82: 1-11.

133. Mak JW, et al. Parasitological and serological surveys for malaria among the inhabitants of an aborigine village and an adjacent Malay village. Acta Trop 1987; 44: 83-89.

134. Greenwood BM, et al. Ethnic differences in the prevalence of splenomegaly and malaria in The Gambia. Ann Trop Med Parasitol 1987; 81: 345-54.

135. Bereczky S, et al. Spleen enlargement and genetic diversity of *Plasmodium falciparum* infection in two ethnic groups with different malaria susceptibility in Mali, West Africa. Trans R Soc Trop Med Hyg 2006; 100: 248-57.

136. Dolo A, et al. Difference in susceptibility to malaria between two sympatric ethnic groups in Mali. Am J Trop Med Hyg 2005; 72: 243-8.

137. Modiano D, et al. Interethnic differences in the humoral response to non-repetitive regions of the *Plasmodium falciparum* circumsporozoite protein. Am J Trop Med Hyg 1999; 61: 663-667.

138. Modiano D, et al. Different response to *Plasmodium falciparum* malaria in west African sympatric ethnic groups. Proc Natl Acad Sci U S A 1996; 93: 13206-11.

139. Modiano D, et al. Humoral response to *Plasmodium falciparum* Pf155/ring-infected erythrocyte surface antigen and Pf332 in three sympatric ethnic groups of Burkina Faso. Am J Trop Med Hyg 1998; 58: 220-4.

140. Modiano D, et al. HLA class I in three West African ethnic groups: genetic distances from sub-Saharan and Caucasoid populations. Tissue Antigens 2001; 57: 128-137

141. Modiano D, et al. The lower susceptibility to *Plasmodium falciparum* malaria of Fulani of Burkina Faso (west Africa) is associated with low frequencies of classic malaria-resistance genes. Trans R Soc Trop Med Hyg 2001; 95: 149-52.

142. Farouk SE, et al. Different antibody- and cytokine-mediated responses to *Plasmodium falciparum* parasite in two sympatric ethnic tribes living in Mali. Microbes Infect 2005; 7: 110-7.

143. van der Pol W, van de Winkel JG. IgG receptor polymorphisms: risk factors for disease. Immunogenetics 1998; 48: 222-32.

144. Rascu A, et al. Clinical relevance of Fc gamma receptor polymorphisms. Ann N Y Acad Sci 1997; 815: 282-295.

145. Ronnelid J, et al. Immune complexes from SLE sera induce IL10 production from normal peripheral blood mononuclear cells by an FcγRII dependent mechanism: implications for a possible vicious cycle maintaining B cell hyperactivity in SLE. Ann Rheum Dis 2003; 62: 37-42.

146. Warmerdam PA, et al. A single amino acid in the second Ig-like domain of the human Fcγ receptor II is critical for human IgG2 binding. J Immunol 1991; 147: 1338-43.

147. Braga É M, et al. Polymorphism in the Fcγ receptor IIA and malaria morbidity. Journal of Molecular and Genetic Medicine 2005; 1: 5-10.

148. Aucan C, et al. High immunoglobulin G2 (IgG2) and low IgG4 levels are associated with human resistance to *Plasmodium falciparum* malaria. Infect Immun 2000; 68: 1252-8.

149. Nasr A, et al. Fcγ receptor IIa (CD32) polymorphism and antibody responses to asexual blood-stage antigens of *Plasmodium falciparum* malaria in Sudanese patients. Scand J Immunol 2007; 66: 87-96

150. Ouma C, et al. Association of Fcγ receptor IIa (CD32) polymorphism with malarial anemia and high-density parasitemia in infants and young children. Am J Trop Med Hyg 2006; 74: 573-577.

151. Sinha S, et al. Polymorphisms of TNF-enhancer and gene for FcγRIIa correlate with the severity of *falciparum* malaria in the ethnically diverse Indian population. Malar J 2008; 7: 13

152. Leoratti FM, et al. Pattern of humoral immune response to *Plasmodium falciparum* blood stages in individuals presenting different clinical expressions of malaria. Malar J 2008; 7: 186

153. Stein MP, et al. C-reactive protein binding to FcγRIIa on human monocytes and neutrophils is allele-specific. J Clin Invest 2000; 105: 369-376.

154. Nimmerjahn F, Ravetch JV. Fcγ receptors: old friends and new family members. Immunity 2006; 24: 19-28

155. Clatworthy MR, et al. Systemic lupus erythematosus-associated defects in the inhibitory receptor FcγRIIb reduce susceptibility to malaria. Proc Natl Acad Sci U S A 2007; 104: 7169-7174

156. Metes D, et al. Expression of functional CD32 molecules on human NK cells is determined by an allelic polymorphism of the FcγRIIC gene. Blood 1998; 91: 2369-2380.

157. Ernst LK, et al. Allelic polymorphisms in the FcγRIIC gene can influence its function on normal human natural killer cells. J Mol Med 2002; 80: 248-257

158. Koene HR, et al. FcγRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell FcγRIIIa, independently of the FcγRIIIa-48L/R/H phenotype. Blood 1997; 90: 1109-14.

159. Omi K, et al. Absence of association between the Fcγ receptor IIIA-176F/V polymorphism and the severity of malaria in Thai. Jpn J Infect Dis 2002; 55: 167-169.

160. Omi K, et al. Fcγ receptor IIA and IIIB polymorphisms are associated with susceptibility to cerebral malaria. Parasitol Int 2002; 51: 361-6.

161. Abrams ET, et al. Malaria during pregnancy and foetal haematological status in Blantyre, Malawi. Malar J 2005; 4: 39.

162. Hurt N, et al. Evaluation of C-reactive protein and haptoglobin as malaria episode markers in an area of high transmission in Africa. Trans R Soc Trop Med Hyg 1994; 88: 182-6.

163. I mrie H, et al. Low prevalence of an acute phase response in asymptomatic children from a malaria-endemic area of Papua New Guinea. Am J Trop Med Hyg 2007; 76: 280-284.

164. Rohner F, et al. Mild riboflavin deficiency is highly prevalent in school-age children but does not increase risk for anaemia in Cote d'Ivoire. Br J Nutr 2007; 97: 970-976

165. Ansar W, et al. Role of C-reactive protein in complement-mediated hemolysis in Malaria. Glycoconj J 2006; 23: 233-240

166. Pied S, et al. C-reactive protein protects against preerythrocytic stages of malaria. Infect Immun 1989; 57: 278-82.

167. Kovacs A, et al. A novel common single nucleotide polymorphism in the promoter region of the C-reactive protein gene associated with the plasma concentration of C-reactive protein. Atherosclerosis 2005; 178: 193-8.

168. Jakobsen PH, et al. Increased concentrations of interleukin-6 and interleukin-1 receptor antagonist and decreased concentrations of beta-2-glycoprotein I in Gambian children with cerebral malaria. Infect Immun 1994; 62: 4374-4379.

169. Jones SA, Rose-John S. The role of soluble receptors in cytokine biology: the agonistic properties of the sIL-6R/IL-6 complex. Biochim Biophys Acta 2002; 1592: 251-263.

170. Sainz J, et al. Genetic variants of IL6 gene promoter influence on C-reactive protein levels but are not associated with susceptibility to invasive pulmonary aspergillosis in haematological patients. Cytokine 2008; 41: 268-278

171. Couper KN, et al. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. PLoS Pathog 2008; 4: e1000004

172. Li C, Corraliza I, Langhorne J. A defect in interleukin-10 leads to enhanced malarial disease in Plasmodium chabaudi chabaudi infection in mice. Infect Immun 1999; 67: 4435-4442.

173. Walther M, et al. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. Immunity 2005; 23: 287-296

174. Torcia MG, et al. Functional deficit of T regulatory cells in Fulani, an ethnic group with low susceptibility to *Plasmodium falciparum* malaria. Proc Natl Acad Sci U S A 2008; 105: 646-651

175. Ouma C, et al. Polymorphic Variability in the Interleukin (IL)-1β Promoter Conditions Susceptibility to Severe Malarial Anemia and Functional Changes in IL-1β Production. J Infect Dis 2008; 198: 1219-1226

176. Odeh M. The role of tumour necrosis factor-alpha in the pathogenesis of complicated *falciparum* malaria. Cytokine 2001; 14: 11-18

177. Sharma S, et al. Association of TNF haplotypes with asthma, serum IgE levels, and correlation with serum TNF-alpha levels. Am J Respir Cell Mol Biol 2006; 35: 488-495

178. Sohail M, et al. Alleles -308A and -1031C in the TNF-alpha gene promoter do not increase the risk but associated with circulating levels of TNF-alpha and clinical features of *vivax* malaria in Indian patients. Mol Immunol 2008; 45: 1682-1692

179. Abraham LJ, Kroeger KM. Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease. J Leukoc Biol 1999; 66: 562-566.

180. Knight JC, et al. A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat Genet 1999; 22: 145-150

181. McGuire W, et al. Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J Infect Dis 1999; 179: 287-290.

182. Hartgers FC, Yazdanbakhsh M. Co-infection of helminths and malaria: modulation of the immune responses to malaria. Parasite Immunol 2006; 28: 497-506

183. Renia L, Potter SM. Co-infection of malaria with HIV: an immunological perspective. Parasite Immunol 2006; 28: 589-595

184. Scott CP, et al. Short report: modulation of *Mycobacterium tuberculosis* infection by *Plasmodium* in the murine model. Am J Trop Med Hyg 2004; 70: 144-148.

185. Troye-Blomberg M, Berzins K. Immune interactions in malaria co-infections with other endemic infectious diseases: implications for the development of improved disease interventions. Microbes Infect 2008

186. Creasey A, et al. Eleven years of malaria surveillance in a Sudanese village highlights unexpected variation in individual disease susceptibility and outbreak severity. Parasitology 2004; 129: 263-271.

187. Giha HA, et al. The epidemiology of febrile malaria episodes in an area of unstable and seasonal transmission. Trans R Soc Trop Med Hyg 2000; 94: 645-651.

188. Dudbridge F. Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol 2003; 25: 115-121

189. Torres A, et al. Molecular analysis of *Mycobacterium tuberculosis* phosphate specific transport system in *Mycobacterium* smegmatis. Characterization of recombinant 38 kDa (PstS-1). Microb Pathog 2001; 30: 289-297

190. Pichyangkul S, et al. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. J Immunol 2004; 172: 4926-4933.

191. Krug A, et al. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur J Immunol 2001; 31: 3026-3037

192. Kadowaki N, Antonenko S, Liu YJ. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c- type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type I IFN. J Immunol 2001; 166: 2291-2295.

193. Bauer M, et al. Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells. J Immunol 2001; 166: 5000-5007.

194. Lazarus R, et al. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 2003; 81: 85-91

195. Boudin C, et al. Possible role of specific immunoglobulin M antibodies to *Plasmodium falciparum* antigens in immunoprotection of humans living in a hyperendemic area, Burkina Faso. J Clin Microbiol 1993; 31: 636-41.

196. Wahlgren M, et al. Anti-*Plasmodium falciparum* antibodies acquired by residents in a holoendemic area of Liberia during development of clinical immunity. Am J Trop Med Hyg 1986; 35: 22-29.

197. Bouharoun-Tayoun H, Druilhe P. Antibodies in falciparum malaria: what matters most, quantity or quality? Mem Inst Oswaldo Cruz 1992; 87 Suppl 3: 229-234.

198. Groux H, Gysin J. Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum*: functional role of IgG subclasses. Res Immunol 1990; 141: 529-42.

199. Eisenhut M. Immunity to blood stages of *Plasmodium falciparum* is dependent on a specific pattern of immunoglobulin subclass responses to multiple blood stage antigens. Med Hypotheses 2007; 69: 804-808

200. Parren PW, et al. On the interaction of IgG subclasses with the low affinity Fcγ RIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2. J Clin Invest 1992; 90: 1537-1546

201. Nasr A, et al. Interethnic differences in carriage of haemoglobin AS and Fcγ receptor IIa (CD32) genotypes in children living in eastern Sudan. Acta Trop 2008; 105: 191-195

202. Yee AM, et al. Association between FcγRIIa-R131 allotype and bacteremic pneumococcal pneumonia. Clin Infect Dis 2000; 30: 25-28.

203. Garraud O, et al. Class and subclass selection in parasite-specific antibody responses. Trends Parasitol 2003; 19: 300-304.

204. Hage FG, Szalai AJ. C-reactive protein gene polymorphisms, C-reactive protein blood levels, and cardiovascular disease risk. J Am Coll Cardiol 2007; 50: 1115-1122

205. Langhorne J, et al. Immunity to malaria: more questions than answers. Nat Immunol 2008; 9: 725-732

206. McGuire W, et al. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature 1994; 371: 508-510.

207. Scopel KK, et al. Factors associated with immunoglobulin G subclass polarization in naturally acquired antibodies to *Plasmodium falciparum* merozoite surface proteins: a cross-sectional survey in Brazilian Amazonia. Clin Vaccine Immunol 2006; 13: 810-813

208. Yu JJ, Gaffen SL. Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity. Front Biosci 2008; 13: 170-177.

209. Rutitzky LI, Lopes da Rosa JR, Stadecker MJ. Severe CD4 T cell-mediated immunopathology in murine schistosomiasis is dependent on IL-12p40 and correlates with high levels of IL-17. J Immunol 2005; 175: 3920-3926.

210. Rutitzky LI, Stadecker MJ. CD4 T cells producing pro-inflammatory interleukin-17 mediate high pathology in schistosomiasis. Mem Inst Oswaldo Cruz 2006; 101 Suppl 1: 327-330.